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(71) Applicants: NOVO NORDISK BIOTECH, INC. [US/US]; 1445 Drew Avenue, Davis, CA 95616-4880 (US). NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors: YAVER, Debbie, Sue; 2809 Albany Avenue, Davis, CA 95616 (US). BROWN, Kimberly, M.; 8322 Windswept Court, Elk Grove, CA 95758 (US). KAUPPINEN, Sakari; Egegade 10, 5tv, DK-2200 Copenhagen N (DK). HALKIER, Torben; Vodroffsvej 4A.7, DK-1900 Frederiksberg C (DK).

(74) Agents: ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., Suite 6400, 405 Lexington Avenue, New York, NY 10174 (US).

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(57) Abstract

The present invention relates to polypeptides having laccase activity and isolated nucleic acid sequences encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides.

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PURIFIED COPRINUS LACCASES AND NUCLEIC ACIDS ENCODING SAME

Background of the Invention

Field of the Invention

The present invention relates to polypeptides having laccase activity and isolated nucleic acid sequences encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides.

Description of the Related Art

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi *et al.* (1992, *Mol. Plant-Microbe Interactions* 5: 119-128) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus *Cryphonectria parasitica*. Kojima *et al.* (1990, *Journal of Biological Chemistry* 265: 15224-15230; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot

basidiomycete *Coriolus hirsutus*. Germann and Lerch (1985, *Experientia* 41: 801; 1986, *Proceedings of the National Academy of Sciences USA* 83: 8854-8858) have reported the cloning and partial sequencing of *the Neurospora crassa* laccase gene. Saloheimo *et al.* (1985, *Journal of General Microbiology* 137:1537-1544; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

It is an object of the present invention to provide polypeptides having laccase activity and nucleic acid constructs encoding these polypeptides.

Summary of the Invention

The present invention relates to isolated polypeptides having laccase activity, obtained from a *Coprinus* strain. The present invention further relates to isolated polypeptides having laccase activity which have: (a) a pH optimum in the range of about 5 to about 9 at 20°C using syringaldazine as a substrate; and (b) an isoelectric point in the range of about 3.7 to about 4.0. The present invention also relates to isolated polypeptides which have an amino acid sequence which has at least 65% identity with the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

The present invention further relates to isolated nucleic acid sequences encoding the polypeptides and to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides.

Brief Description of the Figures

Figure 1 illustrates the nucleotide sequence and the deduced amino acid sequence of the *Coprinus cinereus lcc1* gene.

Figure 2 illustrates the nucleotide sequence and the deduced amino acid sequence of the *Coprinus cinereus lcc3* gene.

Figure 3 illustrates the nucleotide sequence and the deduced amino acid sequence of the *Coprinus cinereus lcc2* gene.

Figure 4 illustrates the construction of plasmid pDSY67.

Figure 5 illustrates a map of plasmid pDSY68.

Figure 6 illustrates the pH activity profiles of recombinant and wild-type *Coprinus cinereus* laccases using (A) syringaldazine and (B) of 2,2'-azinobis-(3-ethybenzthiazoline-6-sulfonic acid (ABTS) as substrates.

Detailed Description of the Invention

Polypeptides Having Laccase Activity

The present invention relates to isolated polypeptides having laccase activity (hereinafter "polypeptides"), obtained from a *Coprinus* strain. The present invention further relates to isolated polypeptides having laccase activity which have:

(a) a pH optimum in the range of about 5 to about 9 at 20°C using syringaldazine as a substrate; and

(b) an isoelectric point in the range of about 3.7 to about 4.0.

The polypeptides preferably have a molecular weight of about 63 kDa (using SDS-PAGE).

In another embodiment, the polypeptides are obtained from a strain of the family Coprinaceae, preferably a *Coprinus* strain, and more preferably a *Coprinus cinereus* strain, *e.g.*, *Coprinus cinereus* IFO 8371 or a mutant strain thereof. In a most preferred embodiment, the polypeptide has the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

The present invention also relates to polypeptides obtained from microorganisms which are synonyms of *Coprinus* as defined by, for example, Webster, 1980, *In* Introduction to the Fungi, Second Edition, Cambridge University Press, New York. Strains of *Coprinus* are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL), *e.g.*, from the American Type Culture Collection, ATCC 12890, 36519, 38628, 42727, 48566 (*Coprinus cinereus*); 15744 (*Coprinus clastophyllus*); 12640, 22314 (*Coprinus* comatus); 46457, 46972 (*Coprinus congregatus*); 48096 (*Coprinus cothurnatus*); 48098 (*Coprinus curtus*); 46973

(*Coprinus disseminatus*); 26829 (*Coprinus domesticus*); 48100 (*Coprinus ephemeroides*); 36567 (*Coprinus fimentarius*); 48097 (*Coprinus gonophyllus*); 20122 (*Coprinus micaceus*); from the Institute for Fermentation (IFO, Osaka, Japan), IFO 8371, 30116 (*Coprinus cinereus*); from Centraalbureau voor Schimmelcultures (CBS; Netherlands) CBS 147.39, 148.39, 175.51 (*Coprinus angulatus*), 147.29 (*Coprinus astramentarius*); 143,39 (*Coprinus auricomus*); 185.52 (*Coprinus* callinus); 159.39, 338.69 (*Coprinus cinereus*); 631.95 (*Coprinus comatus*); 629.95 (*Coprinus friesii*); 627.95 (*Coprinus plicatilis*); 628.95 (*Psathyrella condolleana*); 630.95 (*Panaeolus papilionaceus*) from Deutsche Sammlung von Mikroorganismenn und Zellkulturen (DSM; Germany) DSM 888 (*Coprinus* radians); 4916 (*Csprinus xanthothrix*); 3341 (*Coprinus sterquilinius*). The invention also embraces polypeptides having laccase activity of other fungi and other members of the family Coprinaceae, for example, laccases from the genera *Podaxis*, *Montagnea*, *Macrometrula*, *Psathyrella*, *Panaeolina*, *Panaeolus*, *Copelandia*, *Anellaria*, *Limnoperdon*, *Panaelopsis*, and *Polyplocium*.

For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted.

The present invention also relates to polypeptides which are encoded by nucleic acid sequences which are capable of hybridizing under standard conditions with an oligonucleotide probe which hybridizes under the same conditions with the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32 as well as a complementary strand thereof or a subsequence thereof (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). Hybridization indicates that the analogous nucleic acid sequence hybridizes to the oligonucleotide probe corresponding to the polypeptide encoding part of the nucleic acid sequence shown in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or a subsequence thereof, under medium to high stringency conditions (for example, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, and either 35 or 50% formamide for medium and high stringencies, respectively), following standard Southern blotting procedures.

The nucleic acid sequences set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or subsequences thereof may be used to identify and clone DNA encoding laccases from other strains of different genera or species according to methods well known in the art. Thus, a genomic or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or subsequences thereof. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify clones or DNA which are homologous with SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or subsequences thereof, the carrier material is used in a Southern blot in which the carrier material is finally washed three times for 30 minutes each using 0.2XSSC, 0.1% SDS at 40°C, more preferably not higher than 45°C, more preferably not higher than 50°C, more preferably not higher than 55°C, even more preferably not higher than 60°C, especially not higher than 65°C. Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a X-ray film.

The present invention also relates to polypeptides which have an amino acid sequence which has a degree of identity to the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33 of at least about 65%, preferably about 70%, preferably about 75%, preferably about 80%, preferably about 85%, more preferably about 90%, even more preferably about 95%, and most preferably about 97%, which qualitatively retain the activity of the polypeptides (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence set forth SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33. The degree of identity between two or more amino acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, *Journal of Molecular Biology* 48:443-453). For purposes of determining the degree of identity between two amino acid sequences for the present invention, the Clustal

method (DNASTAR, Inc., Madison, WI) is used with an identity table, a gap penalty of 10, and a gap length of 10.

The amino acid sequences of the homologous polypeptides differ from the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33 by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine and histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine and valine), aromatic amino acids (such as phenylalanine, tryptophan and tyrosine) and small amino acids (such as glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, *e.g.*, by H. Neurath and R.L. Hill, 1979, *In*, *The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

The present invention also relates to polypeptides having immunochemical identity or partial immunochemical identity to the polypeptides having laccase activity which are native to *Coprinus cinereus* IFO 8371. A polypeptide having immunochemical identity to the polypeptide native to *Coprinus cinereus* IFO 8371 means that an antiserum containing antibodies against the antigens of the native polypeptide reacts with the antigens of the other polypeptide in an identical fashion such as total fusion of precipitates, identical precipitate morphology, and/or identical electrophoretic mobility using a specific immunochemical technique. A further explanation of immunochemical identity is described by Axelsen, Bock,

and Krøll, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 10. Partial immunochemical identity means that an antiserum containing antibodies against the antigens of the native polypeptide reacts with the antigens of the other polypeptide in an partially identical fashion such as partial fusion of precipitates, partially identical precipitate morphology, and/or partially identical electrophoretic mobility using a specific immunochemical technique. A further explanation of partial immunochemical identity is described by Bock and Axelsen, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 11. The immunochemical properties are determined by immunological cross-reaction identity tests by the well-known Ouchterlony double immunodiffusion procedure. Specifically, an antiserum against the polypeptide of the invention is raised by immunizing rabbits (or other rodents) according to the procedure described by Harboe and Ingild, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 23, or Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically pages 27-31). Monoclonal antibodies may be prepared, *e.g.*, according to the methods of E. Harlow and D. Lane, editors, 1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York. Purified immunoglobulins may be obtained from the antiserum, *e.g.*, by ammonium sulfate precipitation, followed by dialysis and ion exchange chromatography (*e.g.*, DEAE-Sephadex).

Homologous polypeptides and polypeptides having identical or partially identical immunological properties may be obtained from microorganisms of any genus, preferably from a bacterial or fungal source. Sources for homologous genes are strains of the family Coprinaceae, preferably of the genus *Coprinus* and species thereof available in public depositories. Furthermore, homologous genes may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The nucleic acid sequence may then be derived by similarly screening a cDNA library of another microorganism. Once a nucleic acid sequence encoding

a polypeptide has been detected with the probe(s), the sequence may be isolated or cloned by utilizing techniques which are known to those of ordinary skill in the art (see, *e.g.*, Sambrook *et al.*, *supra*).

As defined herein, an "isolated" polypeptide is a polypeptide which is essentially free of other non-laccase polypeptides, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

Nucleic Acid Sequences

The present invention also relates to isolated nucleic acid sequences obtained from a *Coprinus* strain, which encode a polypeptide of the present invention. In a preferred embodiment, the nucleic acid sequence encodes a polypeptide obtained from *Coprinus cinereus* and in a more preferred embodiment, the nucleic acid sequence is obtained from *Coprinus cinereus* IFO 8371, *e.g.*, the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32. The present invention also encompasses nucleic acid sequences which encode a polypeptide having the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33, which differ from SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, respectively, by virtue of the degeneracy of the genetic code.

As described above, the nucleic acid sequences may be obtained from microorganisms which are synonyms of *Coprinus* as defined by Webster, 1980, *supra*.

The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic sequences of the present invention from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR). See, *e.g.*, Innis *et al.*, 1990, *A Guide to Methods and Application*, Academic Press, New York. The nucleic acid sequence may be cloned from a strain of the *Coprinus* producing the polypeptide, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence.

The term "isolated nucleic acid sequence" as used herein refers to a nucleic acid sequence encoding a polypeptide of the present invention which is isolated by standard

cloning procedures used in genetic engineering to relocate the nucleic acid sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The present invention also relates to nucleic acid sequences which have a nucleic acid sequence which has a degree of identity to the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or subsequences thereof of at least about 65%, preferably about 70%, preferably about 75%, preferably about 80%, preferably about 85%, more preferably about 90%, even more preferably about 95%, and most preferably about 97%, which encode an active polypeptide. The degree of identity between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, *Journal of Molecular Biology* 48:443-453). For purposes of determining the degree of identity between two nucleic acid sequences for the present invention, the Clustal method (DNASTAR, Inc., Madison, WI) is used with an identity table, a gap penalty of 10, and a gap length of 10.

Modification of the nucleic acid sequence encoding the polypeptide may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source. For example, it may be of interest to synthesize variants of the polypeptide where the variants differ in specific activity, thermostability, pH optimum, or the like using, *e.g.*, site-directed mutagenesis. The analogous sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding part of SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, *e.g.*, a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of

nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2:95-107.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *Science* 244:1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for laccase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, *e.g.*, de Vos *et al.*, 1992, *Science* 255, 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224:899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309, 59-64).

Polypeptides of the present invention also include fused polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

The present invention also relates to nucleic acid sequences which are capable of hybridizing under standard conditions with an oligonucleotide probe which hybridizes under the same conditions with the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, a subsequence thereof, or its complementary strand (Sambrook *et al.*, *supra*). Hybridization indicates that the analogous nucleic acid sequence hybridizes to the oligonucleotide probe corresponding to the polypeptide encoding part of the nucleic acid

sequence shown in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32 under standard conditions.

The amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33 or a partial amino acid sequence thereof may be used to design an oligonucleotide probe, or a gene encoding a polypeptide of the present invention or a subsequence thereof can also be used as a probe, to isolate homologous genes of any genus or species. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 40 nucleotides in length. Longer probes, preferably no more than 1200 nucleotides in length, can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{1}H, biotin, or avidin). A PCR reaction using the degenerate probes mentioned herein and genomic DNA or first-strand cDNA from a *Coprinus cinereus* can also yield a *Coprinus cinereus* laccase-specific product which can then be used as a probe to clone the corresponding genomic or cDNA.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence of the present invention operably linked to one or more control sequences capable of directing the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence of the present invention. The term "coding sequence" as defined herein is a sequence which is transcribed into mRNA and translated into a polypeptide of the present invention when placed under the control of the

above mentioned control sequences. The boundaries of the coding sequence are generally determined by a translation start codon ATG at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

An isolated nucleic acid sequence encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleic acid sequence encoding a polypeptide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleic acid sequences utilizing cloning methods are well known in the art.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of the nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, a polyadenylation sequence, a propeptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcription and translation control sequences which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, the *Streptomyces coelicolor* agarase gene (*dagA*), the *Bacillus subtilis* levansucrase gene (*sacB*), the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the *Bacillus licheniformis* penicillinase gene

(*penP*), the *Bacillus subtilis xylA* and *xylB* genes, and the prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:3727-3731), as well as the *tac* gene (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in Sambrook *et al.*, 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium oxysporum* trypsin-like protease (as described in U.S. Patent No. 4,288,627, which is incorporated herein by reference), and hybrids thereof. Particularly preferred promoters for use in filamentous fungal host cells are the TAKA amylase, NA2-tpi (a hybrid of the promoters from the genes encoding *Aspergillus niger* neutral α-amylase and *Aspergillus oryzae* triose phosphate isomerase), and *glaA* promoters.

In a yeast host, useful promoters are obtained from the *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* galactokinase gene (GAL1), the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP), and the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8:423-488. In a mammalian host cell, useful promoters include viral promoters such as those from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus, and bovine papilloma virus (BPV).

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes encoding *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), or *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*. Terminator sequences are well known in the art for mammalian host cells.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence which is functional in the host cell of choice may be used in the present invention.

Preferred leaders for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase and *Aspergillus oryzae* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene, the *Saccharomyces cerevisiae* alpha-factor, and the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, and *Aspergillus niger* alpha-glucosidase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Molecular Cellular Biology* 15:5983-5990. Polyadenylation sequences are well known in the art for mammalian host cells.

The control sequence may also be a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of the polypeptide which can direct the expressed polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the laccase relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide coding region may be obtained from a glucoamylase or an amylase gene from an *Aspergillus* species, a lipase or proteinase gene from a *Rhizomucor* species, the gene for the α-factor from *Saccharomyces cerevisiae*, an amylase or a protease gene from a *Bacillus* species, or the calf preprochymosin gene. However, any signal peptide coding region capable of directing the expressed laccase into the secretory pathway of a host cell of choice may be used in the present invention.

An effective signal peptide coding region for bacterial host cells is the signal peptide coding region obtained from the maltogenic amylase gene from *Bacillus* NCIB 11837, the *Bacillus stearothermophilus* alpha-amylase gene, the *Bacillus licheniformis* subtilisin gene, the *Bacillus licheniformis* beta-lactamase gene, the *Bacillus stearothermophilus* neutral proteases genes (*nprT*, *nprS*, *nprM*), and the *Bacillus subtilis* PrsA gene. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57:109-137.

An effective signal peptide coding region for filamentous fungal host cells is the signal peptide coding region obtained from *Aspergillus oryzae* TAKA amylase gene, *Aspergillus*

niger neutral amylase gene, the *Rhizomucor miehei* aspartic proteinase gene, the *Humicola lanuginosa* cellulase gene, or the *Rhizomucor miehei* lipase gene.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding regions are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding region, which codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the *Bacillus subtilis* alkaline protease gene (*aprE*), the *Bacillus subtilis* neutral protease gene (*nprT*), the *Saccharomyces cerevisiae* alpha-factor gene, or the *Myceliophthora thermophilum* laccase gene (WO 95/33836).

The nucleic acid constructs of the present invention may also comprise one or more nucleic acid sequences which encode one or more factors that are advantageous in the expression of the polypeptide, *e.g.*, an activator (*e.g.*, a trans-acting factor), a chaperone, and a processing protease. Any factor that is functional in the host cell of choice may be used in the present invention. The nucleic acids encoding one or more of these factors are not necessarily in tandem with the nucleic acid sequence encoding the polypeptide.

An activator is a protein which activates transcription of a nucleic acid sequence encoding a polypeptide (Kudla *et al.*, 1990, *EMBO Journal* 9:1355-1364; Jarai and Buxton, 1994, *Current Genetics* 26:2238-244; Verdier, 1990, *Yeast* 6:271-297). The nucleic acid sequence encoding an activator may be obtained from the genes encoding *Bacillus stearothermophilus* NprA (*nprA*), *Saccharomyces cerevisiae* heme activator protein 1 (*hap1*), *Saccharomyces cerevisiae* galactose metabolizing protein 4 (*gal4*), and *Aspergillus nidulans* ammonia regulation protein (*areA*). For further examples, see Verdier, 1990, *supra* and MacKenzie *et al.*, 1993, *Journal of General Microbiology* 139:2295-2307.

A chaperone is a protein which assists another polypeptide in folding properly (Hartl *et al.*, 1994, *TIBS* 19:20-25; Bergeron *et al.*, 1994, *TIBS* 19:124-128; Demolder *et al.*, 1994, *Journal of Biotechnology* 32:179-189; Craig, 1993, *Science* 260:1902-1903; Gething and

Sambrook, 1992, *Nature* 355:33-45; Puig and Gilbert, 1994, *Journal of Biological Chemistry* 269:7764-7771; Wang and Tsou, 1993, *The FASEB Journal* 7:1515-11157; Robinson *et al.*, 1994, *Bio/Technology* 1:381-384). The nucleic acid sequence encoding a chaperone may be obtained from the genes encoding *Bacillus subtilis* GroE proteins, *Aspergillus oryzae* protein disulphide isomerase, *Saccharomyces cerevisiae* calnexin, *Saccharomyces cerevisiae* BiP/GRP78, and *Saccharomyces cerevisiae* Hsp70. For further examples, see Gething and Sambrook, 1992, *supra*, and Hartl *et al.*, 1994, *supra*.

A processing protease is a protease that cleaves a propeptide to generate a mature biochemically active polypeptide (Enderlin and Ogrydziak, 1994, *Yeast* 10:67-79; Fuller *et al.*, 1989, *Proceedings of the National Academy of Sciences USA* 86:1434-1438; Julius *et al.*, 1984, *Cell* 37:1075-1089; Julius *et al.*, 1983, *Cell* 32:839-852). The nucleic acid sequence encoding a processing protease may be obtained from the genes encoding *Saccharomyces cerevisiae* dipeptidylaminopeptidase, *Saccharomyces cerevisiae* Kex2, and *Yarrowia lipolytica* dibasic processing endoprotease (*xpr6*).

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems would include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and the *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the polypeptide would be placed in tandem with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present invention may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such

as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. A frequently used mammalian marker is the dihydrofolate reductase gene. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. A selectable marker for use in a filamentous fungal host cell may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), *trpC* (anthranilate synthase), and glufosinate resistance markers, as well as equivalents from other species. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* markers of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* marker of *Streptomyces hygroscopicus*. Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243, where the selectable marker is on a separate vector.

The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in the cell independent of the genome of the cell.

The vectors of the present invention may be integrated into the host cell genome when introduced into a host cell. For integration, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell

by non-homologous recombination. These nucleic acid sequences may be any sequence that is homologous with a target sequence in the genome of the host cell, and, furthermore, may be non-encoding or encoding sequences.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, pACYC184, pUB110, pE194, pTA1060, and pAMß1. Examples of origin of replications for use in a yeast host cell are the 2 micron origin of replication, the combination of CEN6 and ARS4, and the combination of CEN3 and ARS1. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, *e.g.*, Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA.* 75:1433).

More than one copy of a nucleic acid sequence encoding a polypeptide of the present invention may be inserted into the host cell to amplify expression of the nucleic acid sequence. Stable amplification of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome using methods well known in the art and selecting for transformants.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. The cell is preferably transformed with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome. "Transformation" means introducing a vector comprising a nucleic acid sequence of the present invention into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell.

Integration of the vector into the host chromosome may occur by homologous or non-homologous recombination as described above.

The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The host cell may be a unicellular microorganism or a non-unicellular microorganism. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus*, a *Bacillus licheniformis*, a *Bacillus subtilis*, or a *Bacillus stearothermophilus* cell. The transformation of a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168:111-115), by using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81:823-829, or Dubnar and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56:209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6:742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169:5771-5278).

The host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or, preferably, a fungal cell. Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection. The fungal host cell may be a yeast cell or a filamentous fungal cell.

"Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (*e.g.*, genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (*e.g.*, genera *Pichia*, *Kluyveromyces* and *Saccharomyces*). The basidiosporogenous yeasts

include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti are divided into two families, Sporobolomycetaceae (*e.g.*, genera *Sorobolomyces* and *Bullera*) and Cryptococcaceae (*e.g.*, genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner. F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics are well known in the art (see, *e.g.*, *Biochemistry and Genetics of Yeast*, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; *The Yeasts*, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.*, editors, 1981).

"Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella* (=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

"Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as

Saccharomyces cerevisiae is by budding of a unicellular thallus and carbon catabolism may be fermentative.

In a preferred embodiment, the fungal host cell is a yeast cell. In a more preferred embodiment, the yeast host cell is a cell of a species of *Candida*, *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Pichia*, or *Yarrowia*. In a most preferred embodiment, the yeast host cell is a *Saccharomyces cerevisiae*, a *Saccharomyces carlsbergensis*, a *Saccharomyces diastaticus*, a *Saccharomyces douglasii*, a *Saccharomyces kluyveri*, a *Saccharomyces norbensis*, or a *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

In another preferred embodiment, the fungal host cell is a filamentous fungal cell. In a more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Myceliophthora*, *Mucor*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*. In an even more preferred embodiment, the filamentous fungal host cell is an *Aspergillus* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Fusarium* cell. In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus oryzae*, an *Aspergillus niger*, an *Aspergillus foetidus*, or an *Aspergillus japonicus* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Fusarium oxysporum* or a *Fusarium graminearum* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78:147-156 or in copending US Serial No. 08/269,449. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153:163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:1920.

Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, *Virology* 52:546).

Methods of Production

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a *Coprinus* strain to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide.

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a host cell under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

In both methods, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, *e.g.*, references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it is recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide. Procedures for determining laccase activity are known in the art and include, *e.g.*, the oxidation of 2,2'-azinobis-(3-ethybenzthiazoline-6-sulfonic acid (ABTS) (Childs *et al.*, 1975, *Biochemical Journal* 145:93-103) or syringaldazine (Bauer *et al.*, 1971, *Analytical Chemistry* 43: 421-425) as substrate.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The recovered polypeptide may then be further purified by a variety of chromatographic procedures, *e.g.*, ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like.

The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Uses

The polypeptides of the present invention may be used in a number of different industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin *et al.*, 1991, *Holzforschung* 45: 467-468; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1993. Laccase is also useful in the copolymerization of lignin with low molecular weight compounds, such as is described by Milstein *et al.*, 1994, *Appl. Microbiol. Biotechnol.* 40: 760-767.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, *Current Opinion in Biotechnology* 3: 261-266, 1992; *Journal of Biotechnology* 25: 333-339, 1992; Hiroi *et al.*, 1976, *Svensk Papperstidning* 5:162-166, 1976. Since the environment in a paper

mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, *e.g.*, in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406; WO 92/18683; WO 92/18687; WO 91/05839; EP 0495836; Calvo, 1991, *Mededelingen van de Faculteit Landbouw-wetenschappen/Rijiksuniversitet Gent.* 56: 1565-1567; Tsujino *et al.*, 1991, *J. Soc. Chem.* 42: 273-282. Laccases of the present invention are particularly useful in oxidation at high pH, *i.e.*, over pH 7, as disclosed in DK 0982/94, the contents of which are incorporated herein by reference. Use of laccase in oxidation of dye precursors for hair dyeing is disclosed in U.S. Patent No. 3,251,742, the contents of which are incorporated herein by reference.

The present laccase can also be used for the polymerization or oxidation of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described by Stutz, Fruit processing 7/93, 248-252, 1993; Maier *et al.*, 1990, *Dt. Lebensmittel-rindschau* 86: 137-142; Dietrich *et al.*, 1990, *Fluss. Obst* . 57: 67-73.

Laccases of the present invention are also useful in soil detoxification (Nannipieri *et al.*, 1991, *J. Environ. Qual.* 20: 510-517; Dec and Bollag, 1990, *Arch. Environ. Contam. Toxicol.* 19: 543-550).

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples

Materials and strains

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Strains used are *Coprinus cinereus* A3387 (IFO 8371), *E. coli* Y1090(ZL) (GIBCO BRL, Gaithersburg, MD), *E. coli* DH10B(ZL) (GIBCO BRL), *E. coli* DH5α (Stratagene, La

Jolla, CA), *Aspergillus oryzae* HowB712, *Aspergillus oryzae* JeRS317, and *Aspergillus oryzae* JeRS316.

Example 1: Purification and characterization of *Coprinus cinereus* laccase

The laccase is initially isolated from *Coprinus cinereus* strain A3387 culture broth by filtration (Propex 23+HSC) and concentration (Filtron 2x10K). The cationic flocculent Magnifloc® 521C (American Cyanamid, Wallingford, CT) is added to the resulting preparation, mixed for 30 minutes, and then centrifuged. This step removes colored substances from the preparation. The supernate is then precipitated with ammonium sulfate (55% saturation) and resuspended twice in ammonium sulfate (40% saturation), which also results in color removal. The resuspension is further concentrated to reduce the volume, and filtered, but not washed out. The concentrate in ammonium sulfate (40% saturation) is then subjected to Butyl ToyoPearl hydrophobic chromatography (Tosoh Corp., Tokyo, Japan) and eluted with an ammonium sulfate gradient from 40% to 0% saturation. Buffer exchange to 20 mM MES pH 6.0 and concentration with an Amicon cell equipped with a membrane of 20,000 molecular weight cut-off is then conducted. The resulting solution is then subjected to Q-Sepharose (Pharmacia, Uppsala, Sweden) anion exchange chromatography (150 ml) in 20 mM MES pH 6.0 with a linear gradient from 0 to 0.4 M NaCl. The sample is finally rechromatographed by HPQ-Sepharose (Pharmacia, Upsala, Sweden) chromatography (50 ml) in 20 mM MES pH 6.0 with a linear gradient from 0 to 0.4 M NaCl. The laccase elutes at 0.25-0.30 M NaCl.

The purified laccase is about 95% pure as determined by SDS-PAGE which shows the laccase as a band of M_w=63,000. Isoelectric focusing shows two dominating bands with pIs of 3.7 and 4.0.

The N-terminal amino acid residue of the purified laccase is blocked. The laccase is therefore reduced, S-carboxymethylated, and digested with Endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN) and with chymotrypsin. The resulting peptides are purified by reversed phase HPLC using a Vydac C18 column (Vydac, Inc., Hesperia, CA) eluted with a linear gradient of either acetonitrile or 2-propanol in 0.1% aqueous trifluoroacetic acid. The

purified peptides are sequenced on an Applied Biosystems 473A Protein Sequencer according to the manufacture's instructions.

Several distinct peptides which result from the protease digestion are listed below. In the following sequences, Xaa represents an indeterminable residue. Peptide 3 apparently encompasses peptide 2. In peptides 4 and 9, residues designated Xaa/Yaa indicate both residues are found at that position. Residues in parentheses are uncertain. Peptide 9 is included in peptide 13.

Peptide 1 (SEQ ID NO:1):
Glu-Val-Asp-Gly-Gln-Leu-Thr-Glu-Pro-His-Thr-Val-Asp-Arg-Leu-Gln-Ile-Phe-Thr-Gly-Gln-Arg-Tyr-Ser-Phe-Val-Leu-Asp-Ala-Asn-Gln-Pro-Val-Asp-Asn-Tyr-Trp-Ile-Arg-Ala

Peptide 2 (SEQ ID NO:2):
Xaa-Xaa-Asp-Asn-Pro-Gly-Pro

Peptide 3 (SEQ ID NO:3):
Phe-Val-Thr-Asp-Asn-Pro-Gly-Pro

Peptides 2 and 3 combined (SEQ ID NO:4):
Phe-Val-Thr-Asp-Asn-Pro-Gly-Pro-Trp

Peptide 4 (SEQ ID NO:5):
Ile/Leu-Asp-Pro-Ala-Xaa-Pro-Gly-Ile-Pro-Thr-Pro-Gly-Ala-(Ala)-Asp-Val

Peptide 5 (SEQ ID NO:6):
Gly-Val-Leu-Gly-Asn-Pro-Gly-Ile

Peptide 6 (SEQ ID NO:7):
Xaa-Phe-Asp-Asn-Leu-Thr-Asn

Peptide 7 (SEQ ID NO:8):
Tyr-Arg-Xaa-Arg-Leu-Ile-Ser-Leu-Ser-Cys-Asn-Pro-Asp-(Trp)-Gln-Phe

Peptide 8 (SEQ ID NO:9):
Ala-Asp-Trp-Tyr

Peptide 9 (SEQ ID NO:10):
Ile-Pro-Ala/Asp-Pro-Ser-Ile-Gln

Peptide 10 (SEQ ID NO:11):
Glu-Ser-Pro-Ser-Val-Pro-Thr-Leu-Ile-Arg-Phe

Peptide 11 (SEQ ID NO:12):
Ala-Gly-Thr-Phe

Peptide 12 (SEQ ID NO:13):
Ser-Gly-Ala-Gln-Ser-Ala-Asn-Asp-Leu-Leu-Pro-Ala-Gly

Peptide 13 (SEQ ID NO:14):
Ile-Pro-Ala-Pro-Ser-Ile-Gln-Gly-Ala-Ala-Gln-Pro-Asx-Ala-Thr

Most of the peptides show considerable homology with portions of the amino acid sequence of a *Polyporus pinsitus* laccase (Yaver *et al.*, 1995, *Applied and Environmental Microbiology* 62: 834-841).

Example 2: RNA isolation

Coprinus cinereus strain A3387 is cultivated at 26°C in FG4 medium comprised of 30 g of soybean meal, 15 g of maltodextrin, 5 g of Bacto peptone, and 0.2 g of pluronic acid per liter. The mycelia are harvested after six days of growth, frozen in liquid N_2, and stored at -80°C. Total RNA is prepared from the frozen, powdered mycelium of *Coprinus cinereus*

A3387 by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M cesium chloride cushion (Chirgwin *et al.*, 1979, *Biochemistry* 18: 5294-5299). Poly(A)+ RNA is isolated by oligo(dT)-cellulose affinity chromatography according to Aviv and Leder (1972, *Proceedings of the National Academy of Sciences USA* 69: 1408-1412).

Example 3: Construction of a cDNA library

Double-stranded cDNA is synthesized from 5 μg of *Coprinus cinereus* poly(A)+ RNA of Example 2 as described by Gubler and Hoffman (1983, *Gene* 25: 263-269) and Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York), except that an oligo(dT)-*Not*I anchor primer, instead of an oligo(dT)12-18 primer, is used in the first strand reaction. After synthesis, the cDNA is treated with Mung bean nuclease (Life Technologies, Gaithersburg, MD), blunt-ended with T4 DNA polymerase (Boehringer Mannheim, Indianapolis, IN), and ligated to non-palindromic *Bst*XI adaptors (Invitrogen, San Diego, CA), using about 50-fold molar excess of the adaptors. The adapted cDNA is digested with *Not*I, size-fractionated for 1.2-3.0 kb cDNAs by agarose gel electrophoresis, and ligated into *Bst*XI/*Not*I cleaved pYES2.0 vector (Invitrogen, San Diego, CA). The ligation mixture is transformed into electrocompetent *E. coli* DH10B cells (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The library consisting of 1 x 10^6 independent clones is stored as individual pools (25,000-30,000 colony forming units/pool) in 20% glycerol at -80°C, and as double stranded cDNA and ligation mixture at -20°C.

Example 4: Generation of a cDNA probe from a *Coprinus cinereus* cDNA using PCR

Three oligonucleotides (sense s1 and s2 and antisense as1) with low codon degeneracy are designed based on two conserved motifs in laccases from *Rhizoctonia*, *Phlebia*, *Polyporus*, and *Coriolus*. The oligos have the following sequences:

s1: 5'-ATI CAc/t TGG CAc/t GGI c/tTI c/tTI-3' (SEQ ID NO:15)

s2: 5'-ATI CAc/t TGG CAc/t GGI TTc/t Ttc/t-3' (SEQ ID NO:16)

as1: 3'-GGI ACC AAa/g a/gAI GTa/g ACa/g GTa/g TAI CT-5' (SEQ ID NO:17)

One μg of plasmid DNA from the *Coprinus cinereus* library pool described in Example 3 is PCR amplified in a thermal cycler according to Frohman *et al.*, 1988, *Proceedings of the National Academy of Sciences USA* 85: 8998-9002 using 500 pmol of each laccase sense primer in two combinations (s1 and as1, s2 and as1) with 500 pmol of the laccase antisense primer, and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus, Branchburg, NJ). Thirty cycles of PCR are performed using a cycle profile of denaturation at 94°C for 1 minute, annealing at 55°C for two minutes, and extension at 72°C for 3 minutes. Analysis of the PCR products reveals a 1.2 kb major PCR product with one primer pair, s1 and as1, whereas the other pair does not amplify any major products. The PCR fragment of interest is subcloned into a pUC18 vector and sequenced according to Siggaard-Andersen *et al.*, 1991, *Proceedings of the National Academy of Sciences USA* 88: 4114-4118. Sequencing of the ends of two PCR subclones in pUC18 reveals a cDNA sequence coding for a laccase polypeptide. In addition to the primer encoding residues, the deduced amino acid sequence aligns with two peptide sequences obtained from the purified wild-type laccase, indicating that PCR has specifically amplified the desired region of a *Coprinus cinereus* laccase cDNA.

Example 5: Subcloning and sequencing of partial cDNAs

The PCR product described in Example 4 is ligated into pCRII using a TA Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Seven subclones are prepared and sequenced using both the M13 universal -21mer oligonucleotide and the M13 -48 reverse oligonucleotide. Nucleotide sequences are determined on both strands by primer walking using *Taq* polymerase cycle-sequencing with fluorescent-labeled nucleotides, and reactions are electrophoresed on an Applied Biosystems Automatic DNA Sequencer (Model 373A, version 2.0.1).

The seven clones based on deduced amino acid sequence and percent identities between them appear to encode for 3 laccases (Table 1). Clones CCLACC4, 8 and 7 are designated as partial cDNAs of *Coprinus cinereus lcc1* (SEQ ID NOS:18 (corresponding to nucleotides 1107-2481 of SEQ ID NO:26) and 19). Clones CCLACC 1, 3 and 11 (pDSY71) are designated as partial cDNAs of *Coprinus cinereus lcc2* (SEQ ID NOS:20 (corresponding to nucleotides 875-2451 of SEQ ID NO:28) and 21). Clone CCLACC 15 (pDSY72) is

designated as a partial cDNA of *Coprinus cinereus lcc3* (SEQ ID NOS:22 (corresponding to nucleotides 1005-2501 of SEQ ID NO:32) and 23). The deduced amino acid sequences of the partial cDNAs of *lcc1*, *lcc2*, and *lcc3* (SEQ ID NOS:19, 21, and 23) are compared to the peptide sequences determined above, and the closest match is found between *lcc1* and the peptide sequences. In order to obtain a full-length clone for heterologous expression of *lcc1* in *Aspergillus oryzae*, a genomic library of *Coprinus cinereus* A3387 is constructed in λZipLox.

Table 1. Percent identities between *Coprinus cinereus* cDNAs

		1	2	3	4	5	6	7
1	CCLACC4		98	100	65	65	65	62
2	CCLACC7			98	65	64	65	63
3	CCLACC8				65	65	65	62
4	CCLACC1					100	99	81
5	CCLACC3						99	81
6	CCLACC11							81
7	CCLACC15							

Example 6: Genomic DNA isolation

A culture of *Coprinus cinereus* A3387 is grown at room temperature for 4 days with shaking at 200 rpm in YEG medium comprised of 0.5% yeast extract and 2% dextrose. Mycelia are harvested through Miracloth (Calbiochem, La Jolla, CA), washed twice with 10 mM Tris-0.1 mM EDTA pH 7.4 buffer (TE) and frozen quickly in liquid nitrogen. DNA is isolated as described by Timberlake and Barnard, 1981, *Cell* 26: 29-37.

Example 7: Preparation of *Coprinus cinereus* genomic library

A genomic library of *Coprinus cinereus* A3387 is constructed using a λZipLox Kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Genomic DNA (~30 μg) is digested with *Tsp*509I (New England Biolabs, Beverly, MA) at 65°C in a total volume of 150 μl in the buffer provided by the supplier. Samples of 30 μl are taken at

3, 5, 7, 8, and 9 minutes and electrophoresed on a 1% agarose preparative gel. Bands of 3 to 8 kb in size are excised from the gel. The DNA is then isolated from the gel slices using a Qiaex Kit (Qiagen, San Diego, CA). The size-fractionated DNA is ligated overnight at room temperature to λZipLox *Eco*RI arms following the protocols provided with the kit. The ligations are packaged into phage using a Giga Pak Gold Packaging Kit (Stratagene, La Jolla, CA), and the packaging reactions are titered using *E. coli* Y1090 cells. A total of 6×10^5 pfu are obtained. The packaging extract is plated to amplify the library, and the titer of the library is determined to be 1×10^{11} pfu/ml. Twenty individual plaques are picked, and the plasmids are excised from the plaques by passage through *E. coli* DH10B. Plasmid DNA is isolated from the cultures and is digested with *Pst*I/*Not*I to determine the percent of molecules in the library which have inserts. Eight of the twenty, or 40% of those tested, have inserts which range in size from 3 to 6 kb.

Example 8: Probe preparation for library screening

A DIG-labeled probe for nonradioactive screening of the library is prepared by PCR using the *Coprinus cinereus* partial *lcc1* cDNA described in Example 5 as a template. The primers used in the reaction are shown below:

5' ACTGCGATGGTCTCCGTGGTC 3' (SEQ ID NO:24)

5' GGGGCCTGGGTTATCGGTGAC 3' (SEQ ID NO:25)

The PCR conditions are 1 cycle at 95°C for 5 minutes, 50°C for 1 minute, and 72°C for 1.5 minutes; 29 cycles each at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; and 1 cycle at 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 3 minutes. The reaction contains 0.1 μg of the *Coprinus cinereus* partial *lcc1* cDNA, 10 μl 10X PCR Buffer (Perkin Elmer, Branchburg, NJ), 5 μl 10X DIG labeling mix (Boehringer Mannheim, Indianapolis, IN), 75 pmol of each primer, and 0.5 unit of *Taq* DNA polymerase (Perkin-Elmer Corp., Branchburg, NJ). A probe concentration of 250 ng/μl is determined after PCR following protocols provided with the Genius Kit (Boehringer Mannheim, Indianapolis, IN).

^{32}P-labeled probes of *Coprinus cinereus lcc2* and *lcc3* partial cDNAs are prepared using a RadPrime Kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions.

Example 9: Genomic library screening

Appropriate dilutions of the λZipLox *Coprinus cinereus* genomic library are plated with *E. coli* Y1090 cells on NZY plates comprised of 0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, and 1% NZ amine pH 7.5 per liter with 0.7% top agarose. The plaques are lifted to Hybond N+ filters (Amersham Co., Amersham, UK) using standard procedures (Sambrook *et al.* 1989, *supra*). The filters are hybridized in Engler Blue hybridization buffer at 65°C for 1 hour. After prehybridization, the DIG labeled probe of Example 8 is added at a final concentration of 3 ng/ml and allowed to hybridize overnight at 65°C. The filters are washed at 65°C twice for 5 minutes in 2XSSC, 0.1% SDS, twice for 15 minutes in 0.5XSSC, 0.1%SDS, and then are processed to detect the hybridized DIG-label using the Genius Kit and Lumi-Phos 530 substrate according to the manufacturer's instructions. Following the detection protocol, film is placed on top of the filters for 2 hours.

For screening of the library using the ^{32}P-labeled probes described in Example 8, filter lifts are prepared as described above, and prehybridized at 65°C in 2XSSPE, 1% SDS, 0.5% nonfat dry milk and 200 μg denatured salmon sperm DNA. After 1 hour prehybridization, the ^{32}P-labeled probes are added to a final concentration of 10^6 cpm/ml and hybridizations are continued overnight at 65°C. The filters are washed twice at 65°C for 15 minutes in 0.2XSSC, 1% SDS, and 0.1% sodium pyrophosphate.

The genomic library is probed with the DIG-labeled fragment of *lcc1*. Approximately 200,000 plaques are screened using the conditions described above, and 9 positive clones are obtained. The plasmids are excised from the clones by passage through *E. coli* DH10B(ZL), and then are characterized by digestion with *Pst*I/*Not*I. All 9 clones contain inserts. Based on the nucleotide sequence of the partial *lcc1* cDNA, the genomic clones which may be *lcc1* genomic clones are determined. All 8 unique clones are digested with *Bam*HI/*Pst*I and *Pst*I/*Bsm*I for which fragments of 205 bp and 382 bp, respectively, are expected (neither *lcc2* nor *lcc3* partial cDNAs contain these fragments). Four of the 8 unique clones contain both predicted fragments. DNA sequencing reactions on all four clones using universal sequencing primers are performed as described in Example 5 to determine which clones are full-length.

The nucleotide sequence of clone 4-19 (pDSY73) is determined completely on both strands and shown to contain the full length *lcc1* gene (Figure 1, SEQ ID NO:26). The

deduced amino acid sequence (Figure 1, SEQ ID NO:27) of the genomic *lcc1* matches 100% with the determined N-terminal sequence (see Example 14) although the predicted signal peptide cleavage site is between A18 and Q19 while the peptide sequence begins 4 residues downstream at S23. The *lcc1* gene contains 7 introns ranging in size from 54 to 77 bp. The deduced protein contains 3 potential N-glycosylation sites (AsnXaaThr/Ser), and the predicted mature protein after removal of the signal peptide is 521 amino acids in length. The percent identities of the Lcc1 protein to other fungal laccases is shown in Table 2. The highest percent identity, 57.8%, is found when compared to the laccase from the unidentified basidiomycete PM1 (Coll *et al.*, 1993, *supra*). When alignments of Lcc1 and other basidiomycete laccases are performed, it appears that Lcc1 may have either a C-terminal extension or a C-terminal peptide that is removed by processing.

The genomic library is also screened with the ^{32}P-labeled probes for the *Coprinus cinereus lcc2* and *lcc3* partial cDNAs. For screening with the *lcc2* probe, approximately 50,000 plaques are hybridized with the probe, and 4 positive clones are obtained. For screening of the library with the *lcc3* probe, approximately 35,000 plaques are probed, and 2 positive clones are obtained. After passage through *E. coli* and isolation of the plasmid DNA, the nucleotide sequence of one of the *lcc3* clones (pDSY100) is determined by primer walking as described in Example 5 (Figure 2, SEQ ID NO:28). The *lcc3* gene contains 13 introns (as indicated by lowercase in Figure 2). The positions of introns 4 through 10 are confirmed from the partial cDNA while the positions of the other 6 introns are deduced based on the consensus sequences found at the 5' and 3' splice sites of fungal introns and by homology of the deduced amino acid sequence (Figure 2, SEQ ID NO:29) to other laccases. The *lcc3* gene encodes for a precursor protein of 517 amino acids. There is one potential N-glycosylation site, and the mature protein after the predicted signal peptide cleavage (indicated by an arrow) is 501 amino acids in length.

From the nucleotide sequences of the 4 positive *lcc2* clones, it is observed that none of the clones are full-length. The clone with the largest insert (CCLACC1-4) is missing the sequence coding for the last approximately 100 amino acids based on homology to other fungal laccases.

Table 2. Percent identities of the *Coprinus cinereus lcc1* to other fungal* laccases

	Cc *lcc1*	Cc *lcc2*	Cc *lcc3*	Tv *lcc1*	Tv *lcc2*	Tv *lcc3*	Tv *lcc4*	Tv *lcc5*	Ch	Pr	PMI	Ab	Nc
Cc*lcc1*													
Cc*lcc2*	59.3												
Cc*lcc3*	57.5	79.6											
Tv*lcc1*	55.5	61.3	59.5										
Tv*lcc2*	55.7	60.9	59.5	79.6									
Tv*lcc3*	57.0	61.0	58.2	62.8	84.6								
Tv*lcc4*	55.5	59.2	58.8	70.3	67.1	61.4							
Tv*lcc5*	54.4	59.3	57.9	71.1	69.1	64.6	76.5						
Ch	55.5	61.5	59.3	91.4	81.4	63.0	70.1	71.3					
Pr	50.3	59.1	57.5	63.3	61.5	62.2	63.9	63.9	64.1				
PMI	57.8	62.8	59.4	79.6	73.7	62.2	69.1	70.1	80.2	65.7			
Ab	40.3	41.7	41.9	43.7	43.1	43.6	44.6	43.1	44.1	42.5	44.4		
Nc	25.3	25.3	24.0	25.1	23.8	24.8	21.9	24.2	25.1	23.0	24.4	25.5	

*Cc=*Coprinus cinereus*; Tv=*Trametes villosa*; Ch=*Coriolus hirsutus*; PM1=unidentified basidiomycete; Pr=*Phlebia radiata*; Nc=*Neurospora crassa*; Ab=*Agaricus bisporus*; lcc=laccase gene.

Example 10: Probe preparation for library screening to obtain the full length *lcc2* gene

A DIG-labeled probe for nonradioactive screening of the library is prepared by PCR using the *Coprinus cinereus lcc2* partial genomic clone as template in order to obtain a full-length clone of *lcc2*. The primers used in the reaction are shown below:

AGCTCGATGACTTTGTTACGG (1868R CCLCC2) (SEQ ID NO:30)

CAGCGCTACTCGTTCGTTCTC (1460 CCLCC2) (SEQ ID NO:31)

The PCR conditions are 1 cycle at 95°C for 1 minute; and 30 cycles each at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The reaction contains 0.1 μg of *Coprinus cinereus lcc2* partial genomic clone (CCLACC1-4), 10 μl of 10X PCR Buffer (Perkin Elmer, Branchburg, NJ), 5 μl of 10X DIG labeling mix (Boehringer Mannheim,

Indianapolis, IN), 75 pmol of each primer, and 0.5 Unit of *Taq* DNA polymerase. The concentration of the DIG-labeled probe is determined using the Genius Kit according to the manufacturer's instructions.

Example 11: Genomic library screening to obtain the full length *lcc2* gene

Appropriate dilutions of the λZipLox *Coprinus cinereus* genomic library prepared as described in Example 7 are plated with *E. coli* Y1090 cells on NZY plates (0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, and 1% NZ amine pH 7.5) with 0.7% top agarose. The plaques are lifted to Hybond N+ filters using standard procedures (Sambrook *et al.*, 1989, *supra*). Filters are prehybridized in Easy Hyb hybridization buffer (Boehringer Mannheim, Indianapolis, IN) at 42°C for 1 hour, and after prehybridization the DIG labeled probe mentioned above is added at a final concentration of 1 ng/ml. The filters and probe are allowed to hybridize overnight at 42°C. The filters are then washed twice at room temperature for 5 minutes in 2XSSC-0.1% SDS and twice at 68°C for 15 minutes in 0.1XSSC-0.1% SDS. The filters are next processed to detect the hybridized DIG-label using the Genius Kit and CSPD Ready-To-Use (Boehringer Mannheim, Indianapolis, IN) as substrate according to the manufacturer's instructions. Following the detection protocol, film is placed on the filters for 20 minutes to 2 hours.

In order to obtain a full-length clone, the genomic library is screened (~42,000 plaques) using a DIG-labeled fragment containing the 3' most 400 bp of the CCLACC1-4 insert. Five positive clones are isolated and purified. Plasmid DNA is excised from all five clones by passage through *E. coli* DH10B. Using a specific primer to the 3' end of the CCLACC1-4 insert in sequencing reactions as described in Example 5, it is determined that only one of the clones (LCC2-5B-1) contains the 3' missing portion of *lcc2* gene. However, further sequencing demonstrates that (LCC2-5B-1) does not contain the whole gene but is missing part of the 5' end. Overlapping the sequences of CCLACC1-4 and CCLACC2-5B-1 yields the sequence of the entire gene (Figure 3, SEQ ID NO:32).

A plasmid pDSY105 containing the full-length *lcc2* genomic clone is constructed by ligating together fragments from the LCC2-5B-1 and CCLACC1-4 clones. Clone LCC2-5B-1 is digested with *Eag*I and *Bgl*II and electrophoresed on a 1% agarose gel. The gel slice

containing the 1.3 kb *EagI/BglII* fragment is excised, and the DNA is isolated using a Spin Bind column (FMC). A PCR reaction is performed to obtain an *EcoRI/BglII* fragment containing the N-terminal half of *lcc2*. The PCR reaction mixture contains 0.1 mg of CCLACC1-4 DNA, 50 pmol each of oligonucleotides 96-0545 and 96-0546, 0.01 mM each of dATP, dCTP, dGTP, and dTTP, and 0.5 U *Taq* DNA polymerase. PCR conditions are 1 cycle at 95°C for 5 minutes, 55°C for 1 minute, and 72°C for 1 minute; and 30 cycles each at 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. The primers used in the reaction are:

96-0545: AGAATTGACTCCACCGACGAA (SEQ ID NO:34)

96-0546: GAATTCTGGCATTCCTGACCTTTGTTC (SEQ ID NO:35)

The desired product of 1.6 kb is subcloned into pCRII using the TA Cloning Kit (Invitrogen, San Diego, CA). Partial nucleotide sequences of the subclones are determined using M13-20 universal and M13 -48 reverse universal primers. The final plasmid is constructed by digesting pBluescript SK- with *EcoRI/EagI* and ligating with the *EagI/BglII* fragment from LCC2-5B-1 and the *BglII/EcoRI* fragment from the pCRII subclone. The resulting subclones are screened by restriction digests, and the desired product is designated pDSY105.

The *lcc2* gene contains 13 introns (indicated by lowercase in Figure 3). The positions of introns 4 through 10 are confirmed from the partial cDNA while the positions of the other 6 introns are deduced based on the consensus sequences found at the 5' and 3' splice sites of fungal introns and by homology of the deduced amino acid sequence (Figure 3, SEQ ID NO:33) to other laccases. The *lcc2* gene encodes for a precursor protein of 517 amino acids in length. There is one potential N-glycosylation site, and the mature protein after the predicted signal peptide cleavage is 499 amino acids in length.

From the alignment of the Lcc1, Lcc2 and Lcc3 predicted mature proteins, it appears that unlike Lcc1 neither Lcc2 nor Lcc3 contains the 23 amino acid extension present on Lcc1. Lcc1 shares 59.3% and 57.5% identity with Lcc2 and Lcc3, respectively (Table 2). When compared to other fungal laccases, Lcc2 and Lcc3 have the highest identity (79.6%) with one another. The percent identities shared with other fungal laccases range from a high of 62.8% for Lcc2 and the basidiomycete PM1 laccase to a low of 21.9% for Neospora Crassa laccase.

Example 12: Construction of pDSY67 and pDSY68 for heterologous expression of *lcc1* in *Aspergillus oryzae*

pDSY67 (Figure 4) and pDSY68 (Figure 5) are constructed for expression of *Coprinus cinereus lcc1* gene. The *Coprinus cinereus lcc1* gene is cloned into the expression vector pKS4 which contains the TAKA promoter, AMG terminator and the *Aspergillus nidulans* pyrG for selection. The *lcc1* gene is inserted as 3 fragments into pKS4 digested with SwaI/NotI to obtain pDSY67 (Figure 4). Sequencing of pDSY67 reveals the presence of 32 extra base pairs between the stop codon and the AMG terminator. pDSY68 is generated by removing the extra thirty-two base pairs. In order to remove the extra base pairs, pDSY67 is digested with *PacI/NotI* and the ends are blunted using T4 DNA polymerase. The blunt-end vector is ligated to itself, and the resulting plasmid pDSY68 is sequenced to confirm the extra base pairs are removed.

Example 13: Transformation of *Aspergillus oryzae*

Aspergillus oryzae strains HowB712, JeRS316, and JeRS317 are grown for 18 hours in YEG medium at 34°C, and protoplasts are generated and transformed as described by Christensen *et al.* (1988, *Biotechnology* 6: 1419-1422). The protoplasts are transformed with 10 μg of either pDSY67 or pDSY68. Transformants are selected on Minimal medium plates containing 1.0 M sucrose. Minimal medium plates are comprised of 6.0 g of $NaNO_3$, 0.52 g of KCl, 1.52 g of KH_2PO_4, 1.0 ml of trace metals solution, 20 g of Nobel Agar (Difco), 20 ml of 50% glucose, 20 ml of methionine (50 g/l), 20 ml of biotin (200 mg/l), 2.5 ml of 20% $MgSO_4$-$7H_2O$, and 1.0 ml of mg/ml streptomycin per liter. The agar medium is adjusted to pH 6.5 prior to autoclaving and then glucose, methionine, biotin, $MgSO_4$-$7H_2O$, and streptomycin are added as sterile solutions to the cooled autoclaved medium and poured into plates. The trace metals solution is comprised of 22 g of $ZnSO_4$-$7H_2O$, 11 g of H_3BO_3, 5 g of $MnCl_2$-$4H_2O$, 5 g of $FeSO_4$-$7H_2O$, 1.6 g of $CoCl_2$-$5H_2O$, 1.6 g of $(NH_4)_6Mo_7O_{24}$, and 50 g of Na_4EDTA per liter.

Example 14: Screening of laccase transformants

Primary transformants are screened first on Minimal medium plates containing 1% glucose as the carbon source and 1 mM 2,2'-azinobis-(3-ethybenzthiazoline-6-sulfonic acid) (ABTS) to test for production of laccase. Transformants producing green zones on the ABTS plates are picked and spore purified before shake flask analysis. For shake flask analysis, the purified transformants are cultivated at 37°C in MY51 medium comprised of 30 g of maltose, 2 g of $MgSO_4$, 10 g of KH_2PO_4, 2 g of K_2SO_4, 2 g of citric acid, 10 g of yeast extract, 0.5 ml of trace metals solution, 1 g of urea, 2 g of $(NH_4)_2SO_4$ pH 6.0 per liter. The trace metals solution is comprised of 14.3 g of $ZnSO_4$-$7H_2O$, 2.5 g of $CuSO_4$-$5H_2O$, 11 g of $NiCl_2$-$6H_2O$, 13.8 g of $FeSO_4$-$7H_2O$, 8.5 g of $MnSO_4$-H_2O, and 3.0 g of citric acid per liter. Samples are taken at various intervals and centrifuged. The supernatants are diluted and assayed using ABTS as a substrate.

Laccase activity is determined by syringaldazine oxidation. Specifically, 60 μl of syringaldazine stock solution (0.28 mM in 50% ethanol) and 20 μl of laccase sample are mixed with 0.8 ml of preheated Britton-Robinson buffer solution and incubated at 20°C. The oxidation is monitored at 530 nm over 5 minutes and activity is expressed as "SOU" μmole syringaldazine oxidized per minute ("SOU"). Britton-Robinson buffers with various pHs are used. ABTS oxidation assays are performed at 20°C using 1 mM ABTS, Britton-Robinson buffers (diluted 1.1-fold) by monitoring ΔA405 in 96-well plates.

For pDSY67, 3, 8, and 64 transformants, which are positive on ABTS, are obtained in *Aspergillus oryzae* JeRS316, JeRS317, and HowB712, respectively. For pDSY68, 34 and 56 transformants, which are positive on ABTS plates, are obtained in JeRS317 and HowB712, respectively. On average >90% of the primary transformants are positive on ABTS plates. All of the transformants are spore purified and tested in shake flask for production of the laccase as described above. Laccase activity assays confirm that the transformants, which are positive on ABTS plates, are indeed producing laccase.

Example 15: Purification and characterization of recombinant *Coprinus cinereus* Lcc1

Aspergillus oryzae JeRS317 (pDSY68, *lcc1*) is inoculated into a 10 liter lab fermentor containing medium comprised of Nutriose, yeast extract, $(NH_4)_2HPO_4$, $MgSO_4$-$7H_2O$, citric

acid, K_2SO_4, $CaCl_2$-H_2O, and trace metals solution and supplemented with $CuSO_4$ and fermented at 31°C, pH 7, 600-700 rpm for 7 days. The broth is then recovered and filtered through cheesecloth.

Cheesecloth filtered broth (pH 7.2, 15 mS) is filtered through Whatman #2 filter paper, then concentrated and washed on a Spiral Concentrator (Amicon) with a S1Y30 membrane (16-fold, 0.8 mS). The broth is frozen overnight at -20°C, thawed the next day, filtered again on Whatman #2 paper, and loaded onto a 120 ml Q-Sepharose XK26 column (Pharmacia, Uppsala, Sweden), pre-equilibrated with 10 mM Tris pH 7.7, 0.9 mS (Buffer A). After loading and washing with Buffer A, a linear gradient with Buffer B (Buffer A plus 2 M NaCl) is applied and the active fractions are eluted around 7% Buffer B. The active fractions are dialyzed in Buffer A and then loaded onto a 40 ml Mono-Q 16/10 (Pharmacia, Uppsala, Sweden) column, pre-equilibrated with Buffer A. The active fractions pass through the column.

The sequential ion-exchange chromatography on Q-Sepharose and Mono-Q yields a recombinant *Coprinus cinereus* laccase preparation with apparent homogeneity by SDS-PAGE analysis. An overall 64-fold purification and a recovery of 23% are achieved.

A molecular weight of 66 kDa for the recombinant laccase is observed by SDS-PAGE analysis, similar to that of wild type laccase. The difference between the observed molecular weight and that derived from the DNA sequence (56 kDa) suggests the laccase is 18% glycosylated. The chromatographic elution pattern of recombinant laccase is very close to that of the recombinant *Myceliophthora thermophila* laccase under the same conditions, where the recombinant *Coprinus cinereus* laccase has a similar pI to the pI of 4.2 for recombinant *Myceliophthora thermophila* laccase, which is also close to the pI of wild type *Coprinus cinereus* laccase (3.7 - 4.0).

Copper (Cu) titration of the purified recombinant laccase with 2,2'-biquinoline is carried out as described by Felsenfeld, 1960, *Archives of Biochemistry and Biophysics* 87: 247-251. Photometric titration with 2,2'-biquinoline gives a Cu to protein (subunit) stoichiometry of 3.4 ± 0.2, indicating the four-Cu oxidase nature of recombinant *Coprinus cinereus* laccase.

The purified recombinant *Coprinus cinereus* laccase shows a UV-visible spectrum with two maxima at 278 and 614 nm. The ratio of absorbance at 280 nm to that at 600 nm is found as 22.

The extinction coefficient for the enzyme is determined by amino acid analysis and the molecular weight derived from the DNA sequence. Amino acid analysis suggests an extinction coefficient of 1.6 l/(g*cm), similar to the predicted value of 1.2.

The redox potential is measured by monitoring the recombinant *Coprinus cinereus* laccase's absorbance change at 600 nm with $K_3Fe(CN)_6$- $K_4Fe(CN)_6$ couple (0.433 V) and with I_2-NaI couple (0.536 V) in 9 mM MES-NaOH pH 5.3 buffer. At pH 5.3, a redox potential of 0.55 ± 0.06 V is observed for the recombinant *Coprinus cinereus* laccase.

The activity of recombinant *Coprinus cinereus* laccase is tested with syringaldazine and ABTS. With syringaldazine as the substrate, recombinant *Coprinus cinereus* laccase shows a LACU/A_{280} of 2.7 or a LACU/mg near 4. The recombinant laccase exhibits a pH activity profile in the pH range from about 4 to about 9 with optimal activity at pH 6 to 7 similar to that of wild type *Coprinus cinereus* laccase (Figure 6A), at which its SOU/A_{280} = 5.6. At pH 5.3, syringaldazine shows a K_m of 26 ± 6 μM and a k_{cat} of 180 ± 20 min^{-1}. With ABTS as the substrate, the recombinant laccase shows a pH activity profile in the pH range from about 2.7 to about 7 with optimal activity at pH 4 similar to wild type *Coprinus cinereus* laccase (Figure 6B). At pH 5.3, a K_m of 23 ± 3 μM and a k_{cat} of 1090 ± 30 min^{-1} are observed for ABTS oxidation. The values for K_m and k_{cat} are determined by fitting initial rates ($v = \Delta A/\Delta t/\Delta e$; Δe: extinction coefficient change), laccase concentration (E), and substrate concentration (S) into $v = k_{cat}*E*S/(K_m + S)$ with the Prizm nonlinear regression software (GraphPad, San Diego, CA). Total amino acid analysis, from which the extinction coefficient is determined, is performed on a HP AminoQuant instrument.

Example 16: N-terminal sequencing

Wild type *Coprinus cinereus* laccase is treated with a number of deblocking agents in order to remove the blocked N-terminus. Buffer exchange of samples is carried out in BioRad's BioSpin (P-6) device. Samples are treated with pyroglutamate aminopeptidase (Boehringer Mannheim, Indianapolis, IN and Sigma, St. Louis, MO), acylamino acid peptidase

(Boehringer Mannheim, Indianapolis, IN), and acylase I (Sigma, St. Louis, MO) with deblocking protocols adapted from manufacturer's recommendations as follows. For pyroglutamate aminopeptidase treatment, a laccase sample is exchanged into 5% glycerol-10 mM EDTA-0.1 M sodium phosphate pH 8, then mixed with dithiothreitol (DTT) to 0.7 mM and horse liver peptidase (Sigma, St. Lois, MO) to 1/216 w/w laccase. The mixture (~6.2 mg/ml in laccase) is divided into three aliquots, of which one is adjusted 1 M urea and another is adjusted 0.5 M guanidine-HCl. Each sample is incubated at 4°C for 16 hours. For acylamino acid peptidase, a laccase sample is exchanged into 0.2 M NH_4HCO_3 pH 7.8, then mixed with EDTA to 1 mM, 2-mercaptoethanol to 1 mM, and peptidase to 1/5 w/w laccase. The mixture (~14 mg/ml in laccase) is divided into three aliquots, of which one is adjusted 0.01% in SDS, one is adjusted 0.08 M in guanidine-HCl, and another is adjusted 0.7 M in urea. Each sample is incubated at 37°C for 20 hours. For treatment with acylase I, a laccase sample is exchanged into 0.1 M sodium phosphate pH 7, then mixed with the acylase to 1/3 w/w of laccase. The mixture (~15 mg/ml in laccase) is incubated at 37°C for 22 hours.

The enzyme-treated laccase samples are concentrated using Amicon's Microcon-10 devices. The concentrated samples are run on SDS-PAGE and electroblotted onto a PVDF membrane of sequencing grade (Novex, San Diego, CA). The PVDF membrane is stained with Coommassie blue R-250 to visualize the treated laccase bands. The PVDF membrane is cut to isolate the pieces containing the individual bands. Several lanes are combined and subjected directly to N-terminal sequencing on an ABI 476 Sequencer using a blot cartridge and liquid TFA delivery.

The purified wild-type *Coprinus cinereus* laccase has a blocked N-terminus. However, treatment with both acylamino acid peptidase and acylase I leads to an identical sequenceable N-terminus. The resulting N-terminal sequence is shown below where it is uncertain whether S represents the actual N-terminus in the mature laccase, as, if this is the case, it would require an unexpected deacylase function by acylamino peptidase.

SVDTMTLTNANVSPDGFTRAGI (SEQ ID NO:36)

Under the conditions described, no deblocking is observed with pyroglutamate aminopeptidase.

Direct N-terminal sequencing of the recombinant *Coprinus cinereus* laccase yields a blocked N-terminus, likely due to the same acylation at a Ser as observed in the wild-type laccase.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604, and given the following accession numbers:

Deposit	Accession Number	Date of Deposit
E. coli DH5α with pDSY71 (*lcc2* partial cDNA in pCRII)	NRRL-B 21495	August 18, 1995
E. coli DH5α with pDSY72 (*lcc3* partial cDNA in pCRII)	NRRL-B 21496	August 18, 1995
E. coli DH10B(ZL) with pDSY73 (*lcc1* genomic clone in pZL)	NRRL-B 21497	August 18, 1995
E. coli DH5α with pDSY100	NRRL B-21589	June 21, 1996
E. coli DH5α with pDSY105	NRRL B-21602	July 11, 1996

Claims

What is claimed is:

1. An isolated polypeptide, having laccase activity, obtained from a *Coprinus* strain.

2. The polypeptide according to claim 1, which is obtained from a *Coprinus cinereus* strain and has (a) a pH optimum in the range of about 5 to about 9 at 20°C using syringaldazine as a substrate; and (b) an isoelectric point in the range of about 3.7 to about 4.0.

3. The polypeptide according to claim 2, which is obtained from *Coprinus cinereus* IFO 8371 or a mutant strain thereof.

4. An isolated polypeptide which has an amino acid sequence which has at least 65% identity with the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

5. An isolated polypeptide which has an amino acid sequence which has at least 70% identity with the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

6. The polypeptide according to claim 5, which has an amino acid sequence set forth in SEQ ID NO:27.

7. The polypeptide according to claim 5, which has an amino acid sequence set forth in SEQ ID NO:29.

8. The polypeptide according to claim 5, which has an amino acid sequence set forth in SEQ ID NO:33.

9. The polypeptide according to claim 4, which is obtained from a fungal strain.

10. The polypeptide according to claim 4, which is obtained from a fungal strain of the family Coprinaceae.

11. An isolated polypeptide which is encoded by a nucleic acid sequence which is capable of hybridizing under medium stringency conditions with (a) the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, (b) its complementary strand, or (c) a subsequence of (a) or (b).

12. An isolated polypeptide according to claim 11, which is encoded by a nucleic acid sequence which is capable of hybridizing under high stringency conditions with (a) the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, (b) its complementary strand, or (c) a subsequence of (a) or (b).

13. An isolated nucleic acid sequence comprising a nucleic acid sequence which encodes the polypeptide of claim 4.

14. The nucleic acid sequence according to claim 13, wherein the nucleic acid sequence encodes a polypeptide obtained from a fungal strain.

15. The nucleic acid sequence according to claim 14, wherein the nucleic acid sequence encodes a polypeptide obtained from a fungal strain of the family Coprinaceae.

16. The nucleic acid sequence according to claim 15, wherein the nucleic acid sequence encodes a polypeptide obtained from *Coprinus cinereus*.

17. The nucleic acid sequence according to claim 16, wherein the nucleic acid sequence encodes a polypeptide obtained from *Coprinus cinereus* IFO 8371.

18. The nucleic acid sequence according to claim 13, wherein the nucleic acid sequence is set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:32, or a subsequence thereof.

19. An isolated nucleic acid sequence comprising a nucleic acid sequence encoding a polypeptide which has an amino acid sequence which has at least 65% identity with the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

20. The nucleic acid sequence according to claim 19, wherein the nucleic acid sequence encodes a polypeptide which has an amino acid sequence which has at least 70% identity with the amino acid sequence set forth in SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:33.

21. The nucleic acid sequence according to claim 13, which is capable of hybridizing under medium stringency conditions with (a) the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, (b) its complementary strand or (c) a subsequence of (a) or (b).

22. The nucleic acid sequence according to claim 13, which is capable of hybridizing under high stringency conditions with (a) the nucleic acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, or SEQ ID NO:32, (b) its complementary strand or (c) a subsequence of (a) or (b).

23. The nucleic acid sequence according to claim 13, which is contained in pDSY73 in *Escherichia coli* NRRL-B 21497.

24. The nucleic acid sequence according to claim 13, which is contained in pDSY100 in *Escherichia coli* NRRL-B 21589.

25. The nucleic acid sequence according to claim 13, which is contained in pDSY105 in *Escherichia coli* NRRL-B-21602.

26. A polypeptide encoded by the nucleic acid sequence according to claim 23.

27. A polypeptide encoded by the nucleic acid sequence according to claim 24.

28. A polypeptide encoded by the nucleic acid sequence according to claim 25.

29. A nucleic acid construct comprising the nucleic acid sequence of claim 13 operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

30. A recombinant expression vector comprising the nucleic acid construct of claim 29, a promoter, and transcriptional and translational stop signals.

31. The vector according to claim 30, further comprising a selectable marker.

32. A recombinant host cell comprising the nucleic acid construct of claim 29.

33. A method for producing the polypeptide of claim 1 comprising (a) cultivating a *Coprinus* strain to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide.

34. A method for producing the polypeptide of claim 1 comprising (a) cultivating a host cell comprising a nucleic acid construct comprising a nucleic acid sequence encoding the polypeptide under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

35. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a laccase of claim 1.

36. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a laccase of claim 1.

37. A method for oxidizing dyes or dye precursors which comprises contacting the dye or dye precursor with a laccase of claim 1.

38. A method of polymerizing or oxidizing a phenolic compound which comprises contacting the phenolic compound with a laccase of claim 1.

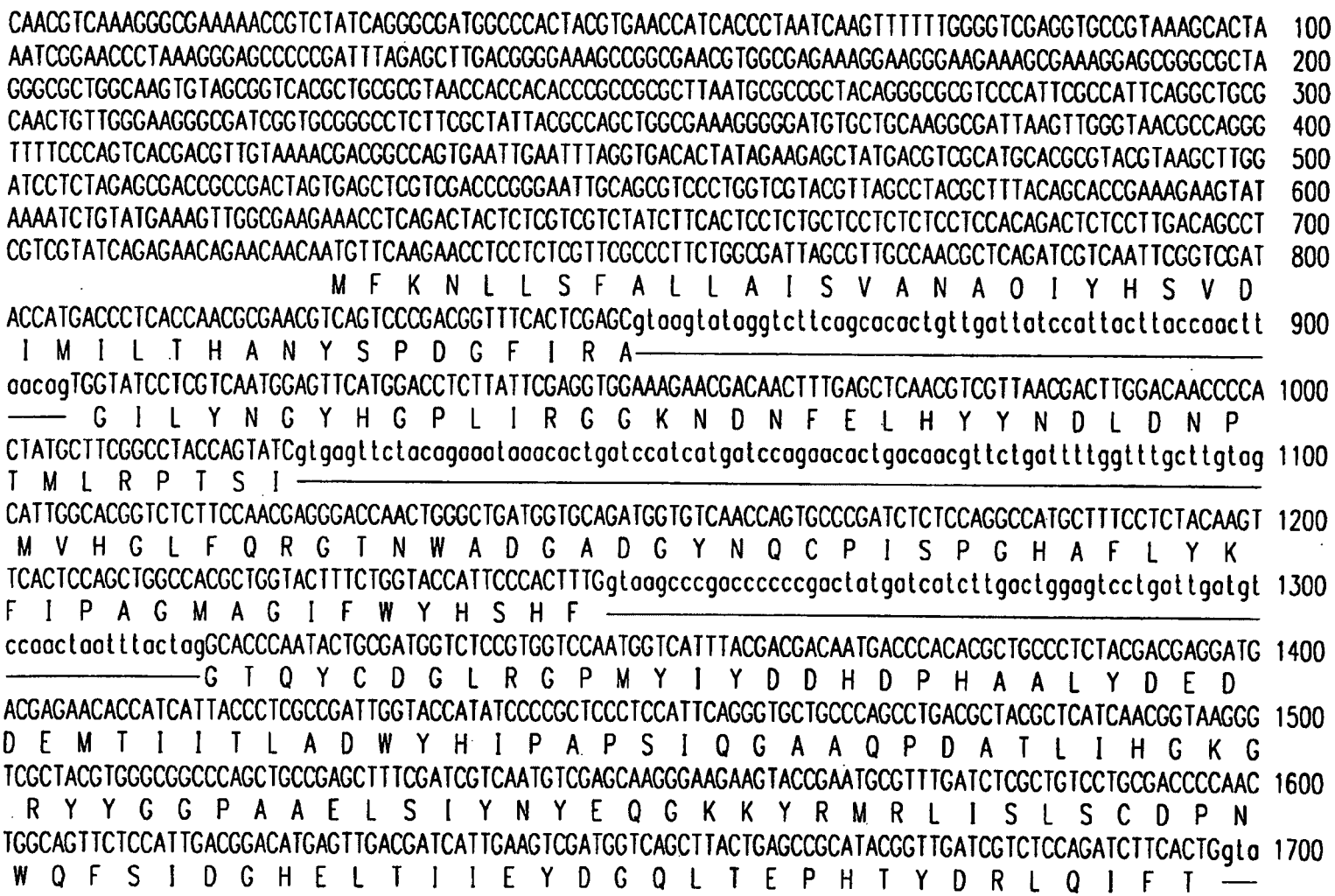

FIG.1A

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agcatlgaaatcggtgtgttccgttgagaaagcacactcacctttaatcagGTCAAAGGTACTCCTTCGTTCTCGACGCCCAACCAGCCCGGTGGACAACT 1800
                                                   G  Q  R  Y  S  F  Y  L  D  A  H  Q  P  Y  D  H
ACTGGATCCGTGCTCAACCCAACAAGGGTCGAAACCGACTTGCTGGTACCTTCGCCAACGGTGTCAACTCGGCCATCCTTCGCTATGCCCGGCGCTGCCAA 1900
T  W  I  R  A  Q  P  H  K  G  R  N  G  L  A  G  T  F  A  N  G  Y  N  S  A  I  L  R  Y  A  G  A  A  N
CGCTGATCCAACCACCTCCGCCAACCCCCAACTCAACGAAGCCGACCTCCATGCTCTCATCGACCCCGCTGCTCCCGCTATCCCCACTCCG 2000
  A  D  P  T  T  S  A  H  P  H  P  A  Q  L  N  E  A  D  L  H  A  L  I  D  P  A  A  P  G  I  P  T  P
GCGCGCTGCAGACGTCAACCTCCGATTCCAATTGGGCTTCAGCGGCGGCTCGATTCACGATTAACGGAACCGCATACGAGAGTCCAAGCGTTCCTACGCTCT 2100
  G  A  A  D  Y  H  L  A  F  Q  L  G  F  S  G  G  R  F  T  I  N  G  T  A  Y  E  S  P  S  Y  P  T  L
TGCAGATTATGAGTGGTGCGCAGAGTGCCGAACGACTTGCTCCCTGCTGGCATCGGTGTATGACTTGCCCAGGAACCAAGTTGTTGAGCTTGTTGTTCCTGC 2200
L  Q  I  M  S  G  A  Q  S  A  M  D  L  L  P  A  G  S  Y  Y  E  L  P  R  H  Q  Y  Y  E  L  Y  Y  P  A
TGCTGTCCTCGGTGGTCCTCATCCTTCCATCTCCACGGTgtacgtcaagttttctttctctctttttttcatgggtggtcaagtgtaccatgagctta 2300
  G  Y  L  G  G  P  H  P  F  H  L  H  G  ____________________________________________________________
ccaaggattgaattgtgtagCATGCGTTCAGTGTCGTCAGGAGTGCAGGCAGCACCTACAACTTTGTCAACCCCGTCAAGCGCGATGTTGTTAGTCT 2400
  ____________H  A  F  S  Y  Y  R  S  A  G  S  S  T  Y  M  F  V  H  P  Y  K  R  D  Y  Y  S  L
TGCTGTTACTGGAGACGAAGTTACCATTCGXTTCGTCACCGATAACCCAGGCCCCGTGGTTCTTCCACTGCCACATTGAATTCCATCTCATGAAgtaagtc 2500
  G  Y  T  G  D  C  Y  T  I  R  F  Y  T  D  N  P  G  P  W  F  F  H  C  H  I  E  F  H  L  M  N_____
ttcatatccatcgttgtatactccagagtctaacccacctccacagCGGCTTGGCGATCGTCTTTGCTGAAGACATGGCGAACACGGTTGATGCTAACAA 2600
  ____________________________________G  L  A  I  Y  F  A  E  D  M  A  H  T  Y  D  A  H  H
CCCACCTGgtacgtcccctcctattgactcaaatactaattccgaagctaacttcggcatcaattacagTCGAGTGGGCCCAGCTTTGCGAGATTTACG 2700
  P  P  ____________________________________________Y  E  W  A  Q  L  C  E  I  T
ATGACCTGCCCCCCTGAGGCGACCTCGATTCAAACCGTTGTGCGTCGCGCTGAGCCCACCGGCTTTTCGGCCAAGTTCCGCAGGGAGGGCTTGTAGATAAT 2800
D  D  L  P  P  E  A  T  S  I  Q  T  Y  Y  R  R  A  E  P  T  G  F  S  A  K  F  R  R  E  G  L
ATTATAGTTGACCAGAGGGCCAGTGGTAGGAGGCTGCTATAGTCAAAGTTGGTCACAGAGGGAAGAGTTAGTCGCAGAAGTCGTTTGAGTACTACTAG 2900
TTATTCATCGTGTTGTTATTTATCGTGGTTGTTACATACTATCGTTATGTGTGCTTGAGTTTGGAATGACAATGTATTTGATTGTCGAGTTG 3000
GAATCCTTGTTGAAGTGCTAGTAACCTTTTGATGGACCTCCGACCTGCCTTTTCCCCGCACTTCCTCGATTGAATATTTGAGCCGCAGGCACCGAATCGAA 3100
CCCACGACCCGCGAATGTCCAAATTCCGGACCGGTACCTGCAGGCGTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATA 3200
GCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA 3300
CTCACATTAATTGCGTTGCGCTCACTG 3327
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FIG.1B

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ACTCACTATAGGGAAAGCTGGTACGCCTGCAGGTACCGGTCCGGAATTCCTTTCACCCCAGATCCTGGTATAGGATAGACCCAGATACTCTTACTAAGGT   100
GGCACGAATGACCGACCGAATCTCGCGAGAAATCTTTCAACTTTTCCAGACACTTGATGAGTCGAAAACAATGCGTTTACCCCTGGAGTTACGGATTGGG   200
TCTCAAGTGACTGTTACAACAAGCGCTCAGGATCCCCTAGTATGTCTAATCGTGACGTCTCTACCGACGCTTGGCGCTCATTGACAGCTATCGCGACAGA   300
TTCTTACATTTTTGTCAACGCCATCCTTTCTCGTTTACGTAGCTTTCTGCTACGGTGCTGTCCTTTGTCAGAGATCCCTCCAGCACGACGATTGATAACG   400
AGATCTCAGTCGACGGAACGGCTCCCTGGACCTGATGCACTTATCCTCTTACTCATTGCAGTCATTACAATCGAGTCTCGTTCGCACGTTGTCACGGAAC   500
GGGACCTGAAAAATGAAGGATATAAACCCCCAAGTGCCGCCCTGAAACTTTCAGACTTTTTGAGTCGACAAGCTCGAGGTCTCCAACATGCAATTGCTTG   600
                                                                                    M  Q  L  L
CCTTCGTCCTCGCTGCTTTACCCCTCGCACGGGCTGCCATTGGCCCTGTTGGCAACCTAGTCATCGCCAACGCGAACGTCTCACCAGACGGCTTCGTTCG   700
A  F  V  L  A  A  L  P  L  A  R  A  A  I  G  P  V  G  N  L  V  I  A  N  A  N  V  S  P  D  G  F  V  R
CTCgtgagtgggcccgcggcctttcaccatttcttttcattaactctcctctgcagGGCTGTCCTTGCCGGCGCTACAGGTACCAGCCTTGAGCACCCAG   800
  S                                                                 A  V  L  A  G  A  T  G  T  S  L  E  H  P
GGCCTGTTATCGTGGGCCAGAAGgtaacactattgacgtcccttggtcagaatccttccttacacccctttatctagGGCGACACTTTCCACATCAATGTC   900
G  P  V  I  V  G  Q  K                                                                 G  D  T  F  H  I  N  V
ATCGATGACCTTACTGACCCCACTATGCTTCGAACAACCAGTATTgtaaagcaaatttgcttggcatccttcaaacttcacactgacgttcatgtcagCA   1000
 I  D  D  L  T  D  P  T  M  L  R  T  T  S  I                                                          H
CTGGCACGGTTTCTTGCAGGAGGGTACAGCTTGGGCCGACGGTCCTGCGGGTGTTACTCAATGCCCCATTGCCCCTGGTCACTCTTTCCTCTATAAGTTC   1100
  W  H  G  F  L  Q  E  G  T  A  W  A  D  G  P  A  G  V  T  Q  C  P  I  A  P  G  H  S  F  L  Y  K  F
CAGGCCAAAAACCAAGCTGGTACCTTCTGGTACCATTCCCACCACAgtgagagcgatgctggtaacggaccttgggtcaatactgactcttgacttacag   1200
 Q  A  K  N  Q  A  G  T  F  W  Y  H  S  H  H
TGTCTCAGTATTGTGACGGCCTGAGAGGCGTCATGGTCGTTTACGATCCCCTAGATCCCCATCGTCACCTgtgcgtacgcctatctatgactctcacctt   1300
M  S  Q  Y  C  D  G  L  R  G  V  M  V  V  Y  D  P  L  D  P  H  R  H  L
cgtactcattccacctacacagGTATGACGTTGATAACGgtaatccttccaacccttacgtctccgctaaagcttacattcaatcttcattgtttcctca   1400
                        Y  D  V  D  N  E
ttttctcagAGAATACTATCATCACGCTCGCGGACTGgtaagcgcgcaaataacctacgaaagttccagtatctgactgttttcagGTATCACGATCCCG   1500
       E  N  T  I  I  T  L  A  D  W                                                          Y  H  D  P
CCCCTTCTGCTGGACTCGTCCCAACCCCCTGGTCGACTTTGATCAATGGCAAGGGCCGTTACCCAGGCGGACCCGTCGTGCCCTTGGCCGTCATTCACGT   1600
A  P  S  A  G  L  V  P  T  P  W  S  T  L  I  N  G  K  G  R  Y  P  G  G  P  V  V  P  L  A  V  I  H  V
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FIG.2A

```
CAGCCGCGGAAAGCGCTACCGCTTCCGCCTCGTCTCCCTTTCGTGCGACCCTAACTATGTATTCTCTATTGACGGTCACACCATGgttcgtaaccctccc  1700
  S  R  G  K  R  Y  R  F  R  L  V  S  L  S  C  D  P  N  Y  V  F  S  I  D  G  H  T  M
atgatccactcctcccctgcctcatattttacgttttgcgactgttagACGGTCATTGAAGTCGATGGTGTCAACCATGAACCGTTGGTTGTCGACCACA  1800
                                                                 T  V  I  E  V  D  G  V  N  H  E  P  L  V  V  D  H
TTCAAATCTTTGCTGGTCAACGGTACTCGTTTGTCTTGAACGCCAACCGGCCCGTCAACAACTACTGGGTCAGGGCTAACCCCAACCTCGGCTCTGTCGG  1900
I  Q  I  F  A  G  Q  R  Y  S  F  V  L  N  A  N  R  P  V  N  N  Y  W  V  R  A  N  P  N  L  G  S  V  G
CTTCGGTGGCGGTATTAATTCCGCAATTCTGCGATATGTTGGAGCTCCTGCCGTCGACCCAACCACCTCCCAATTGCCTTTCAGCAACCCACTCCTCGAG  2000
  F  G  G  G  I  N  S  A  I  L  R  Y  V  G  A  P  A  V  D  P  T  T  S  Q  L  P  F  S  N  P  L  L  E
ACCAACTTGCACCCTCTCGTAAATCCTGCTGCACCTGGCGGCCCTTCCCCCGGTGACGTCGATGTCGCCATCAACCTGGATATCTTGTTCGACGTCTCAA  2100
  T  N  L  H  P  L  V  N  P  A  A  P  G  G  P  S  P  G  D  V  D  V  A  I  N  L  D  I  L  F  D  V  S
TCCTCAAGTTCACTGTCAACGGTGCTACCTTCGATGAACCACCCGTTCCGGTCCTTCTCCAGATTTTGAGCGGTGCACATACCGCCTCATCTCTTCTCCC  2200
I  L  K  F  T  V  N  G  A  T  F  D  E  P  P  V  P  V  L  L  Q  I  L  S  G  A  H  T  A  S  S  L  L  P
CTCTGGCAGCGTCTACACTCTTCCCCCTAACAAGGTCATTGAGCTCACTATTCCCGGTGGTGGTATCGGTGCTCCTgtaggtctttcttcttcatctttc  2300
  S  G  S  V  Y  T  L  P  P  N  K  V  I  E  L  T  I  P  G  G  G  I  G  A  P
tctcgatctcgatggtgttcactcactatttgaaaccagCACCCCATCCATCTTCACGGCgtgagtatccatccgttaagcttcattaagtcccatgctg  2400
                                                H  P  I  H  L  H  G
accgtttgacagCATACCTTCAAGGTTGTCCGTAGCGCAGGCAGCTCGACTTACAACTTCGTCAATCCCGTTGAGCGAGATGTTGTCAACGTTGGTCAAG  2500
                  H  T  F  K  V  V  R  S  A  G  S  S  T  Y  N  F  V  N  P  V  E  R  D  V  V  N  V  G  Q
CTGGCGACAATGTCACCATTCGATTCGTCACTGATAATGCTGGTCCCTGGATTCTTCACTGgtgcgctatttctttaggcattcaacgtgtcagagtctt  2600
A  G  D  N  V  T  I  R  F  V  T  D  N  A  G  P  W  I  L  H  C
acccccgttcttttcagCCACATTGACTGGCATTTGGTTTTgtaagttcacgttttgacgcatcaggcgaatggtactctaacttcctccaGGGGCCTGT  2700
                     H  I  D  W  H  L  V  L                                                    G  L
CTGTCGTCTTCGCGGAAGATGTCCCCACCATCGATAGCTCCGTTCAACCTCgtaagttctgcgtgcctctgctcgatatcatttggctgacttcttggct  2800
S  V  V  F  A  E  D  V  P  T  I  D  S  S  V  Q  P
ttagCCGCCTGGCATGATCTGTGCCCCATCTATGACGCTCTTCCCCCCGGCACGAGGTAATCTCGCCCATGACATACTGGCACGGTATGACTTGGACAGG  2900
   P  A  W  H  D  L  C  P  I  Y  D  A  L  P  P  G  T  R
TTACGGAAATCAAAGTAAATGTTGGATAAGAAGAATAACA  2940
```

FIG.2B

```
TGAAGGAGAATCCCTCGAAGTGGAATTTTCTTTCCAGAAGATGCAATCTGGTTTTGTCTCATCCATTTTTGTGACGTTTACTCACCATTTCGAATCTAGG   100
ATCGTTCGCCGATTTGCTCATATCTTTGCGACCACTCAATATTGCTTTACGTACCCCCTCGTGAGAGGCACAAATGCATTCCTTGCGATGCCCGATTCCA   200
ATCTCAATGCAGGTACGTCCCTGGTTTCATACCAATGCGTGTTTTGGACTGGCATTCCTGACCTTTGTTCCGGTTGACGTTTCTAGTTATTTCGTGTGAC   300
CTGTATGATTAATCGTACAGCCTGAATCTTGTCCTCAAAGTGCACAAATTAGGGCTCAAGCTACCAGGCGAGGCAGGTATAAAGCGCTCTACTCTCCATC   400
CGACGTTCCCCACTCACCACCAGCCGGCTGAGTTCACCCGTTCTTGAAACTCGTTATGTTGCTTTTAGCGACTGCTCTCGCTACATCCCTCTTACCTTTC   500
                                                    M  L  L  L  A  T  A  L  A  T  S  L  L  P  F
GTGCTGGGAGCCATTGGCCCCAGTACCAACCTTGTCGTCGCGAACAAGGTCATCGCTCCCGACGGCTTCAGTCGATCgtgagcctttctgtggactgga   600
 V  L  G  A  I  G  P  S  T  N  L  V  V  A  N  K  V  I  A  P  D  G  F  S  R  S
cgcttcttcagtgactgatcatgtcgcagTGCTGTCCTCGCTGGCGCTACCCAGCCAACGGTGCAGTTCCCTGGCCCCGTCATTCAAGGGAATAAGgtag   700
                                A  V  L  A  G  A  T  Q  P  T  V  Q  F  P  G  P  V  I  Q  G  N  K
gcagatttcaaccgtttcctgtcacatcatgttgagtctttgtagAACAGTTTCTTTGCGATCAACGTCATTGACGCTCTGACCGACCCCACTATGCTGA   800
                                                    N  S  F  F  A  I  N  V  I  D  A  L  T  D  P  T  M  L
GGACTACGAGTATCgtaagtcagttctattgatgctgcgatcagcggaagctcaccatcttttaacagCACTGGCACGGCATGTTCCAAAGGGGGACTGC   900
R  T  T  S  I                                                       H  W  H  G  M  F  Q  R  G  T  A
CTGGGCTGATGGTCCTGCTGGCGTCACCCAATGCCCTATTTCTCCAGGGCATTCGTTCTTGTACAAGTTCCAGGCTCTTAACCAAGCCGGTACTTTCTGG  1000
  W  A  D  G  P  A  G  V  T  Q  C  P  I  S  P  G  H  S  F  L  Y  K  F  Q  A  L  N  Q  A  G  T  F  W
TACCACTCCCATCACGgtaactacaatctatctgtactgacgtgacgatgttgactcagtcattctcagAATCGCAATATTGTGACGGTTTGCGTGGGGC  1100
  Y  H  S  H  H                                                       E  S  Q  Y  C  D  G  L  R  G  A
TATGGTCGTATATGACCCAGTCGACCCACATCGCAACTTgtgagcatcctttacttttattcccaaggaagccatcagtctaatgacttgccatttagGTA  1200
  M  V  V  Y  D  P  V  D  P  H  R  N  L                                                             Y
TGACATTGACAACGgtatgtaacctccggcgtttggtcgtcttgtgatccgcagttcaccttgttttacagAGGCCACGATCATTACGCTCGCAGACTGg  1300
  D  I  D  N                                                          E  A  T  I  I  T  L  A  D  W
taagaatctaattactttcgattaccttcgagcatccctaactcggggcccttctgttcgccagGTATCACGTCCCTGCTCCCTCTGCAGGTCTCGTTCC  1400
                                                                      Y  H  V  P  A  P  S  A  G  L  V  P
CACCCCAGATTCCACGCTTATCAACGGTAAGGGCCCGGTATGCTGGTGGCCCTACCGTACCTCTCGCGGTCATTTCTGTAACCCGAAACCGACGATACCGG  1500
  T  P  D  S  T  L  I  N  G  K  G  R  Y  A  G  G  P  T  V  P  L  A  V  I  S  V  T  R  N  R  R  Y  R
```

FIG.3A

```
TTCCGCCTTGTTTCCCTTTCATGCGATCCTAATTATGTATTCTCTATCGATGGGCATACCATGgtacgcactagttcccatccctgtaaaacgggtgctg   1600
  F  R  L  V  S  L  S  C  D  P  N  Y  Y  V  F  S  I  D  G  H  T  M
acgacgtgtatcatccttagACTGTTATTGAGGTCGACGGAGTTAACGTCCAACCTCTCGTTGTCGACTCGATCCAGATCTTCGCAGGTCAGCGCTACT   1700
                    T  V  I  E  V  D  G  V  N  V  Q  P  L  V  V  D  S  I  Q  I  F  A  G  Q  R  Y
CGTTCGTTCTCAACGCCAACCGCCCCCGTCGGCAACTACTGGGTGCGAGCCAACCCCAACATCGGTACTACGGGCTTCGTCGGTGGAGTCAATTCTGCGAT   1800
S  F  V  L  N  A  N  R  P  V  G  N  Y  W  V  R  A  N  P  N  I  G  T  T  G  F  V  G  G  V  N  S  A  I
TCTGCGCTATGTGGGCGCCTCCAATACAGACCCCACTACCACCCAAACTCCTTTCAGCAACCCTCTCCTTGAGACCAATCTCCACCCCTTGACCAACCCT   1900
  L  R  Y  V  G  A  S  N  T  D  P  T  T  T  Q  T  P  F  S  N  P  L  L  E  T  N  L  H  P  L  T  N  P
GCTGCTCCTGGCTTGCCTACCCCAGGTGCGCTCGACGTCGCGATCAACCTTAACACGGTATTCGATTTCAGTAGTCTCACCTTCTCCGTTAACGGAGCCA   2000
A  A  P  G  L  P  T  P  G  G  V  D  V  A  I  N  L  N  T  V  F  D  F  S  S  L  T  F  S  V  N  G  A
CTTTCCATCAACCGCCCCGTCCCTGTCTTGCTTCAGATCATGAGCGGTGCACAGACTGCCCAGCAGCTTCTCCCTCCGGTTCGGTCTACGTCCTTCCCCG   2100
T  F  H  Q  P  P  V  P  V  L  L  Q  I  M  S  G  A  Q  T  A  Q  Q  L  L  P  S  G  S  V  Y  V  L  P  R
TAACAAACTCATCGAGCTTTCTATGCCTGGAGGCTCCACTGGCAGTCCCgtaagtcttaattgtcttcatttccaacaagtcggtgattaacgctggatc   2200
  N  K  V  I  E  L  S  M  P  G  G  S  T  G  S  P
attcgctgacagCATCCCTTCCATCTCCACGGTgtatgtaggcctctgtctgatctcattcggaagcgttactgacggtgcttctttgtttcgatctgat   2300
        H  P  F  H  L  H  C
agCACGAATTTGCTGTGTCGTCAGAAGCCCGCCGCAGCTTCGCACCTACAACTTCGCCAACCCGGTACGCAGGGATGTCGTGAGTGCCGGTGTTGCTGGTGACAA   2400
  H  E  F  A  V  V  R  S  A  G  S  S  T  Y  N  F  A  N  P  V  R  R  D  V  V  S  A  G  V  A  G  D  N
CGTCACCATTCGATTCCGTACCGATAACCCTGGACCATGGATTCTCCATTGgtgcgtcaagtcatcgtcctcgtgctgaattgattgtctaaccaagata   2500
  V  T  I  R  F  R  T  D  N  P  G  P  W  I  L  H  C
tcacatactagCCATATCGACTGGCACCTTGTTTTgtaagtcttcgcttcttccagacgtgattaacttttactgatcgcgatgatgggaatacagGGGC   2600
        H  I  D  W  H  L  V  L                                                                    G
TTGGCTGTAGTGTTCGCTGAGGACGCTCCTACTGTTGCAACCATGGATCCCCCTCgtgagtagcgcccgtgcttttgaggagttgtgaaacccgagctca   2700
L  A  V  V  F  A  E  D  A  P  T  V  A  T  M  D  P  P
acgtgaaacgttttccactttacagCTGCTTGGGACCAACTTTGCCCGATCTACGATGCTCTCCCTCCCAACACATAAGTCGTTCAATTCAAGGCTGTTG   2800
                    P  A  W  D  Q  L  C  P  I  Y  D  A  L  P  P  N  T
```

FIG.3B

ACGTGAAGGGAGCCAAGAAGGAAAGTAAGAGAAAGGCAGTCACATCCCGTCGGTTTGCCTCTGAAATATCGATTAATCACGCTTTTTATCACTTGTAATTA 2900
TCTTTCTTTGTTACAGTGGCTCTTTGACCCTGGCTTCCAGTGCGTTAGAGTCGATAATAATAGCCAATTCTCTACTTTTTAGGCAGATTTTTTAGGCAGGGC 3000
TGTGGTACGCTTTATATTAAGTTAAAAGAGCACCAATAATGTCGCCCTCAGCTGGGCTCTTGTCGGCCGACTAGCTCAGTTGGTTAGAGCGTCGTGCTAA 3100
TAACGCGAAGGTCTTGGGTTCGATCCCCACGTTGGCCAGTAGCCCCCTTTTTGTTAATCCTGGCACTTTCCTGTTCCTACTAACCCTTTTGAGAGTCCAG 3200
AAAAATCACCATGACTTAATTTTTTTCTTTTTCATAGAAGTCCTGGAAGGGTAAGGAAGTGATATAACTAGATGACCCAACATTCAGTGCTGGTCGTCAGAT 3300
CGAGGTGTCTTTTTCGACCAATCGAAGCATTCGATCCAATTGCGCCTGCCTGTCCGCAGCATCTTCGAACGGCGAAGGACTGTCGAAGAA 3400
CGTTACGTACGCGCGGATTGTCAGTTTACGAAGGCGAGGAAACCCCATTGAGAGTAGATCGTCAAGCGTCTTCCATTGGCCCAGGTCCACATTCAGATCG 3500
CAGCCGATTTGAACGATAGGGATGATATTGAGTCCTCCAGAACGTTCTGTCCCTGCATCAAAGCGA 3566

FIG.3C

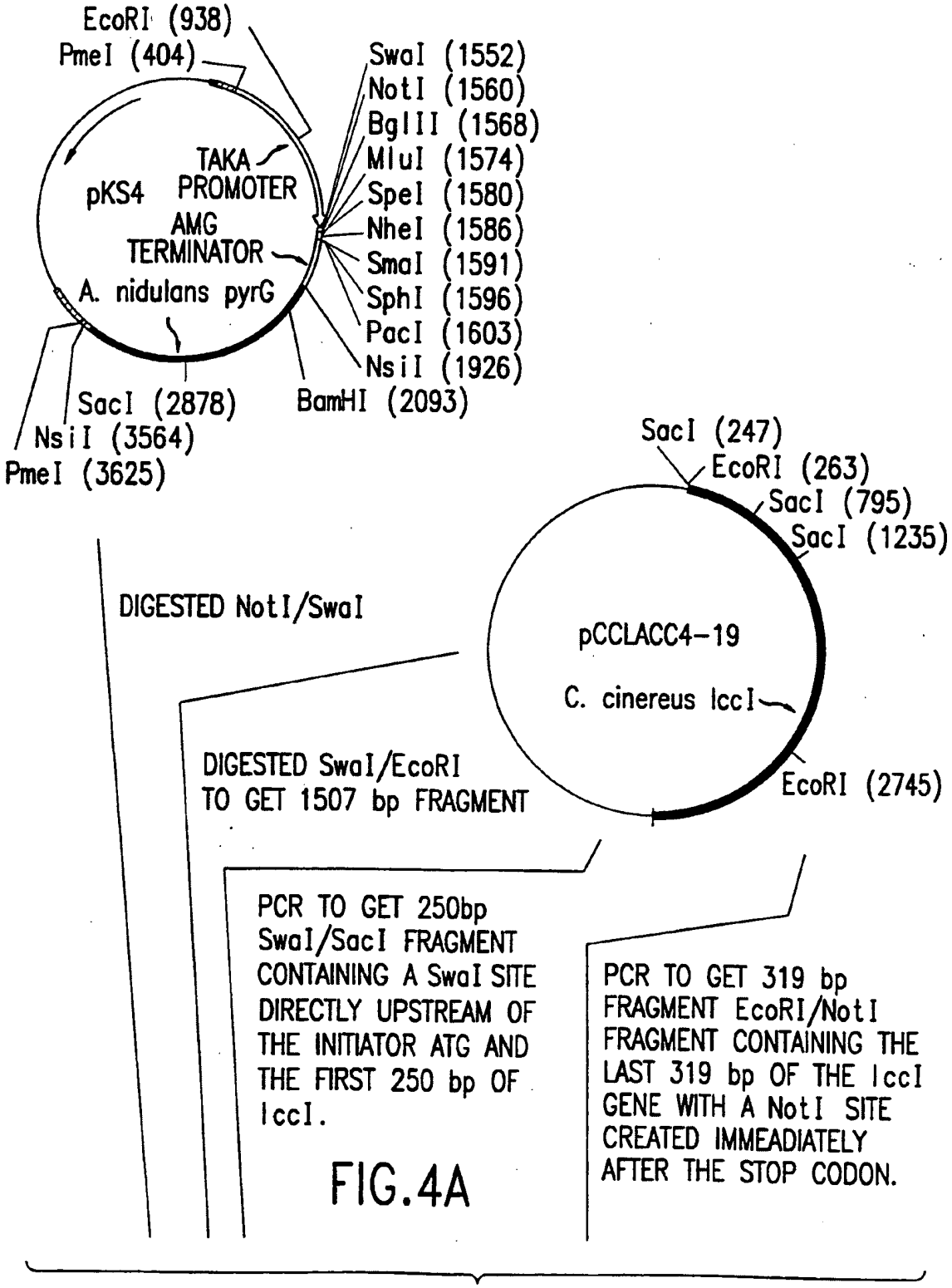

FIG.4A

TO FIG.4B

FROM FIG.4A

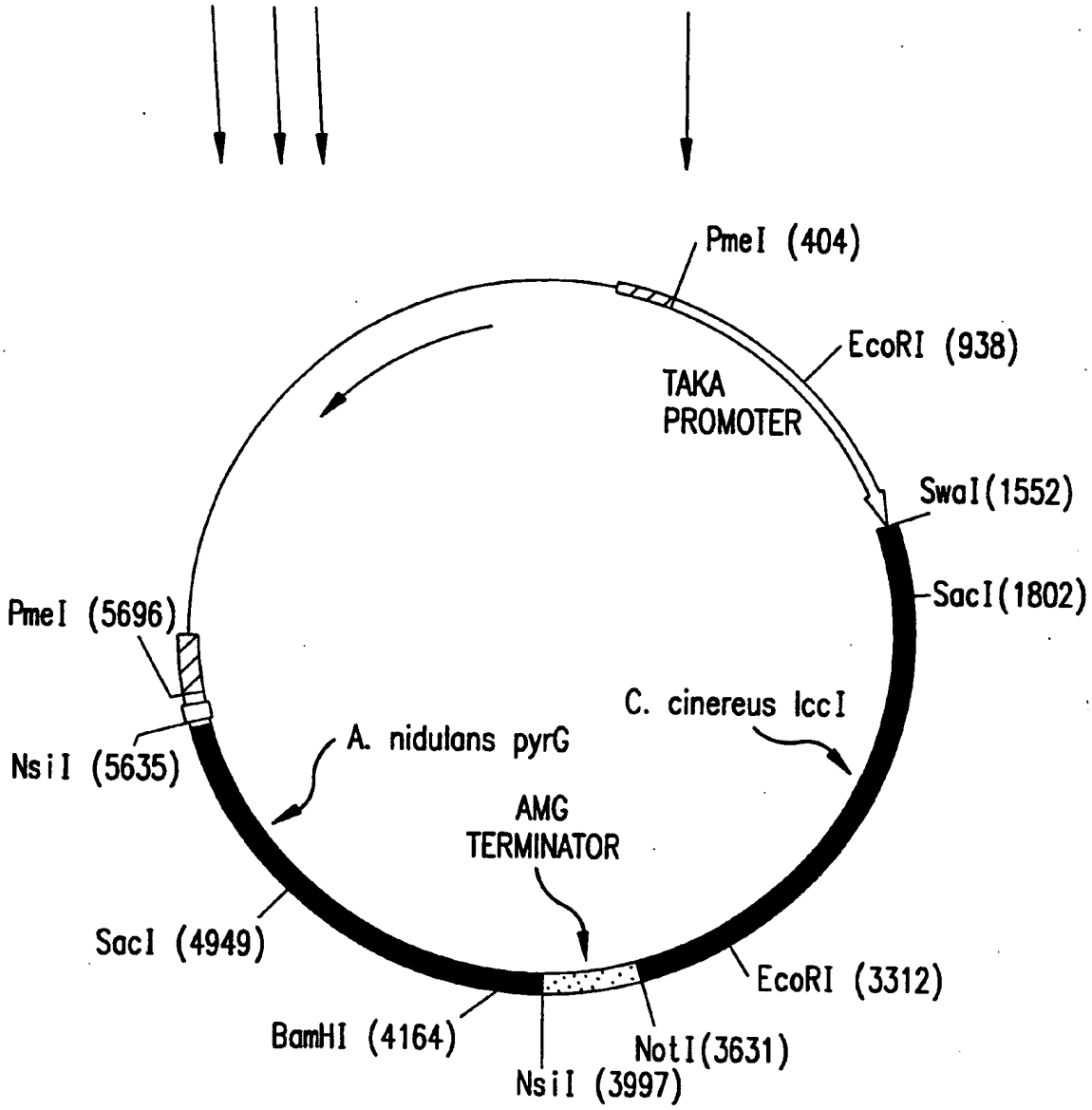

FIG4B

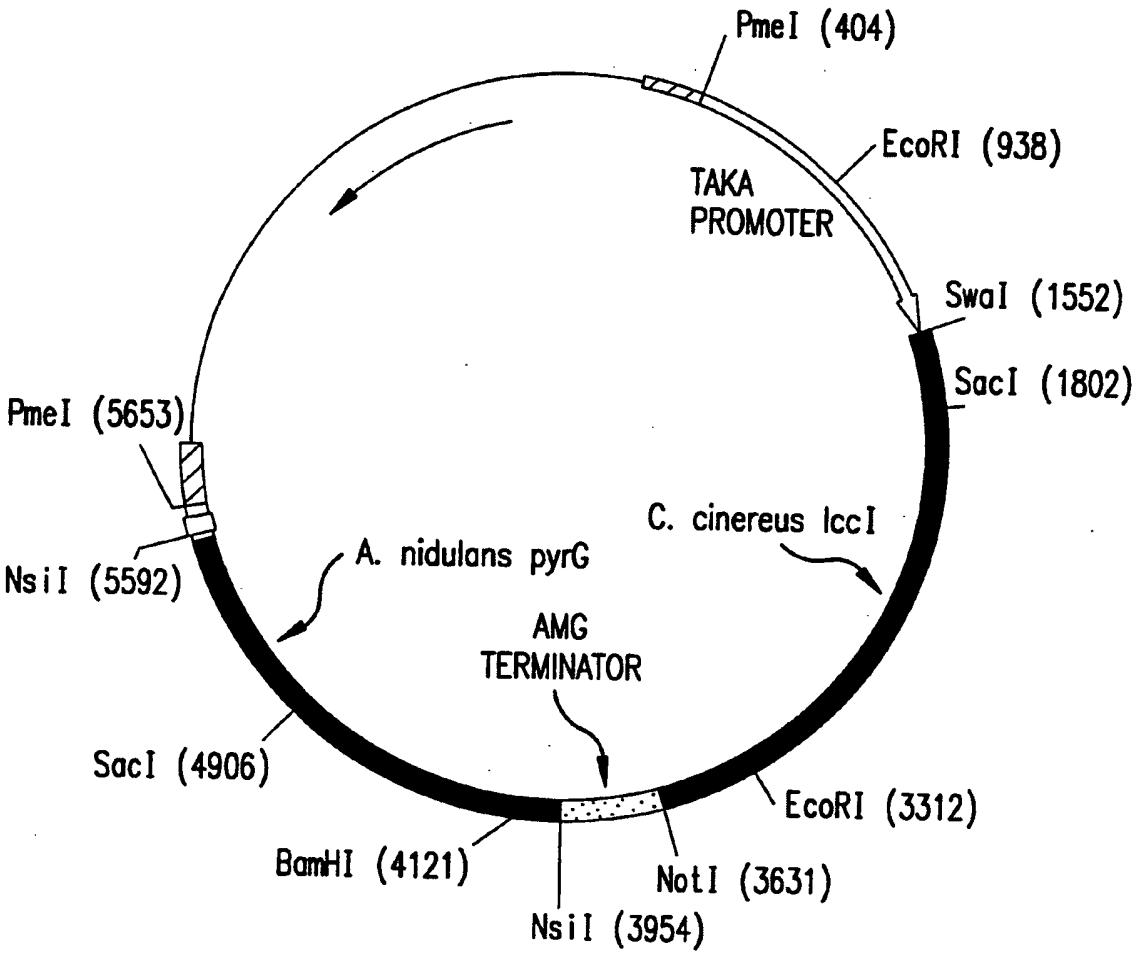
PmeI (404)
EcoRI (938)
TAKA
PROMOTER
SwaI (1552)
SacI (1802)
PmeI (5653)
C. cinereus IccI
NsiI (5592)
A. nidulans pyrG
AMG
TERMINATOR
SacI (4906)
EcoRI (3312)
BamHI (4121)
NotI (3631)
NsiI (3954)

FIG.5

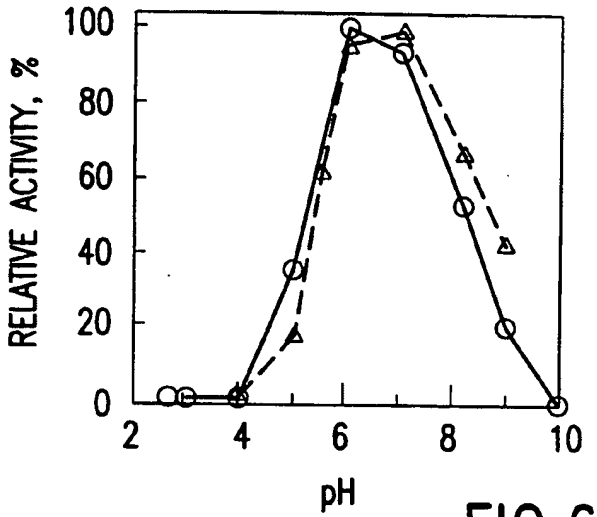
RELATIVE ACTIVITY, %
100
80
60
40
20
0
2
4
6
8
10
pH

FIG.6A

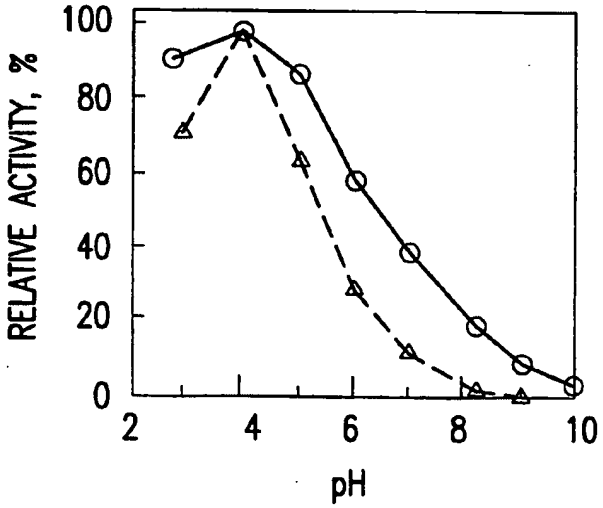
RELATIVE ACTIVITY, %
100
80
60
40
20
0
2
4
6
8
10
pH

FIG.6B